The role of B cells in the amplification and in the regulation of transplant rejection.

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Awarding institution:
King's College London

Title: The role of B cells in the amplification and in the regulation of transplant rejection.

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The role of B cells in the amplification and in the regulation of transplant rejection.

A thesis submitted to King's College London for the degree of Doctor of Philosophy

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October 2012

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Declaration

I, Rowa Yousef Alhabbab, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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October 2012

Abstract

B cells are known to contribute to and/or influence immune response trough a variety of mechanisms. Regulatory B cells (Bregs) have recently discovered and their role in regulating autoimmune diseases has been demonstrated in different murine models. In the context of transplantation B cells are generally thought to promote graft rejection through the production of alloantibody and their capacity to efficiently present alloantigens. However, recent clinical trials with B cell depletion have suggested that B cells contribute to the regulation of immune responses to grafts. In addition the evidence that renal transplant patients that are tolerant to the graft have an expansion of B cells with a regulatory phenotype further confirm this idea.

The hypothesis tested during this PhD is that murine regulatory B cells, defined as transitional-2 marginal zone precursor (T2-MZP), are contributing to transplantation tolerance. The aims of this project are: i) to investigate whether T2-MZP can transfer transplantation tolerance; ii) to analyse whether the expression of Galectin-1 (Gal-1), known to be a functional molecule for Tregs, is necessary for T2-MZP to suppress; iii) to study whether an increase in T2-MZP is observed in tolerant mice like what has been shown in tolerant transplant patients.

The results obtained during this PhD have demonstrated that T2-MZP purified from naïve mice were unable to regulate the immune responses to graft antigens both *in vitro* and *in vivo*. However, their regulatory capacity was observed when T2-MZP were purified from mice kept in the conventional (CV) area of the animal house although IL-10 was not involved in their regulatory

capacity. A difference in the gut flora between mice kept in the Specific-pathogen-Free (SPF) and CV areas was observed and maybe responsible for the differences in the T2-MZP functions. Moreover, Gal-1 was confirmed to be expressed by B cells and T2-MZP isolated from Gal-$1^{-/-}$ mice kept in the CV area lost the ability to suppress *in vitro* $CD4^+$ T cell activation and to prolong skin graft survivals, suggesting that Gal-1 has a functional role in B cell suppressive function. One of the hypotheses was that the absence of Gal-1 influences the response of B cells to gut. Gal-$1^{-/-}$ B cells showed reduced IL-10 production and increased up-regulation of activation markers in response to a variety of Toll-like receptor ligands in comparison to wild-type B cells, and showed differences in the percentage of P38, ERK phosphorylation and NF-κB translocation. Finally, in trying to mimic the findings with renal transplant patients, mice were rendered tolerant to skin transplants using *in vivo* anti-CD40L (MR1) and donor splenocyte transfusion (DST). The number of T2-MZP increased in these mice and these cells were suppressive *in vitro* and, as before, their regulatory capacity did not appear to correlate with IL-10 production. Once transferred *in vivo* T2-MZP isolated from tolerant mice, and not animal rejecting an allograft, induced prolongation of skin transplants onto naïve recipients.

These observations indicate that T2-MZP play a role in transplant tolerance, they need to be “primed” either during tolerance induction protocol or by interaction with gut flora and that Gal-1 is a functional molecule for B cells and T2-MZP B cells.

Acknowledgements

I would like to thank God for the help and support that I found during my PhD. I also would like to thank my family especially my parents and Fahad Aboouf for all their help, time, patience and support.

My thanks to Paul Blair, Giovanna Lombardi and Robert Lechler for their great help, supervision, and their guidance all the way.

Thanks for all the people working in the immunoregulation lab for making my PhD such a wonderful experience and for their support especially Lesley smyth.

Finally, I would like to thank my great friends for their great support especially Heba massri and Niloufar safinia.

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Chapter 1- Introduction

1.1 Transplantation & Rejection

1.1.1 General introduction

Transplantation is currently the treatment of choice for end stage organ failure [1,2]. Damage to the transplanted organ is initiated by ischemia/reperfusion injury associated with the transplant procedure [1,2]. These injuries lead to the activation of the adaptive immune system, which will result in either acute or chronic rejection [1,2]. Transplanted organs can be rejected by the recipient's adaptive immune system through direct, indirect and semi-direct pathways [1]. When grafts are rejected by the direct pathway allo-MHC class I or class II, expressed by donor antigen-presenting cells (APCs), is directly recognized by recipient T cells ($CD4^+$ and $CD8^+$ T cells). This pathway is mainly associated to acute rejection [1]. Once the APC transplanted with the graft disappeared from the circulation, recipient APCs that migrate to the graft, take up and process alloantigens and then display them in the context of self MHC to CD4 and CD8 T cells in the peripheral lymphoid organs (the indirect pathway). This pathway contributes to chronic rejection [1]. Subsequently, the graft gets destroyed via the activated CD4 and CD8 T cells [1]. In the semi-direct pathway, membrane components (MHC) of donor APCs are transferred to recipient APCs via cell-cell contact or via exsome fusion and presented on the recipient APCs both as intact MHC promoting direct pathway and as processed peptide promoting the indirect pathway [1,2]. This pathway allows the presentation of alloantigens on the same APC via direct and indirect pathways [1,2]. The existence of this pathway suggests that the direct pathway never goes to sleep [1,2].

1.1.2 Types of rejection

Hyper-acute rejection

Hyper-acute rejection is an immediate rejection, which is mainly mediated by previously existing antibodies against donor antigens [1]. The presence of alloantibody in the recipient is usually due to prior blood transfusion, pregnancy or earlier transplant [1]. In this type of rejection the binding of the pre-existing alloantibodies to transplanted organ blood vessel endothelial cells, and the subsequent activation of the complement system, results in inflammation and blood coagulation that cause rapid rejection [1,3].

Acute rejection

Acute rejection is the result of recipient T cell recognition of donor MHC on donor DCs that have migrated from the graft to the draining lymph node [3]. Once T cells are activated they move from the lymph nodes to the graft and initiate the rejection largely through cytotoxic T cells [3]. This rejection mainly occurs via the direct pathway, but the indirect pathway can also be involved [3]. This process of rejection requires one day to a few weeks to occur [3].

Chronic rejection

Chronic rejection occurs within months to years post-transplant [4]. Chronic rejection can be due to alloantigen-dependent events or/and antigen-independent factors [5]. Alloantigen-dependent chronic rejection is mainly a consequence of early episodes of acute rejection that lead to chronic rejection [6]. Ongoing episodes of acute rejection result in a continuously activated immune response that may gradually accelerate the process of indirect

alloantigen presentation that might in turn increase the magnitude of the rejection process [7]. Antigen-independent factors include injuries associated with transplantation, such as ischemia/reperfusion injury [6]. Ischemia/reperfusion injury activates resident macrophages to release inflammatory cytokines resulting in endothelial cell (EC) activation [8]. Activated EC mediate the expression of several molecules such as chemokines, adhesion molecules and MHCs on the donor graft EC [8,9,10]. This results in leukocyte recruitment, activation and infiltration into the allograft [8,11,12].

1.1.3 Cells involved in transplant rejection

Innate immunity

T cells are the key player in allograft acute rejection [13,14,15]. However, it is now well established that prior to alloantigen recognition by T cells pro-inflammatory mediators are released into the allograft [13,14,15]. This initial response against the allograft is driven by the innate immune system and not adaptive immune system [13,14,15]. Dendritic (DCs) cells, macrophages, neutrophils and NK cells are innate immune system cells that detect pathogen derived molecules from inherently non-sterile grafts, such as skin, and molecules released from damaged and stressed host tissue through Pathogen-recognition receptors (PRRs) [16,17,18]. PRRs are comprised of a number of different receptors, notably including Toll-like receptors (TLRs), which detect conserved sequences commonly associated with bacterial or viral molecules [16,17,18]. Upon activation of cells through their PRRs a cascade of signaling occurs leading to the activation of the transcription factor nuclear factor (NF)-κB and activator protein-1 (AP-1) [19], and the production of chemokines, pro-

inflammatory cytokines and up-regulation of MHC I, MHC II and co-stimulatory molecules on the surface of APCs [19,20].

A) Dendritic cells

In the absence of danger signals and activating antigens, DCs would remain immature in the periphery [22,23,24]. Although transplantation triggers DCs maturation the stimuli responsible for this are not yet known [21]. Once DCs become activated they mature and produce inflammatory cytokines and up-regulate their expression of their co-stimulatory molecules and chemokine receptors, which induce their migration into secondary lymphoid organs, where they stimulate T cell responses [22,23,24]. It has been reported that the interaction between immature DCs and T cells results in T cells anergy or apoptosis [25]. Given this fact, many researchers have taken an interest in using immature or toleragenic DCs (DCs that deliver inadequate co-stimulatory signals while presenting antigens to antigen-specific T cells [25]) as a therapy to induce peripheral tolerance [22,23,24].

B) Macrophages

Although macrophages are not involved in direct initiation of allograft recognition, they mediate allograft damage through various mechanisms such as the release of pro-inflammatory cytokines, reactive nitrogen and oxygen species, phagocytosis of necrotic debris, and amplifying T cell responses via antigen presentation [26,27].

C) Neutrophils

Neutrophils migrate into transplanted organs within hours of surgery as a result of the trauma caused by the transplant procedure. Neutrophils damage the tissue via a variety of cytotoxic and pro-inflammatory mechanisms such as the release of cytokines and chemokines including IL-1, IL-12, TNF-α, CXCL8 and VEGF [28].

D) Natural Killer cells

Despite the fact that NK cells are not able to cause allograft rejection on their own, they can amplify the magnitude of graft rejection at early stages of the alloresponse and help allo-reactive T cells and DCs exert their functions [29,30,31,32].

E) Complement

Complement can be activated through three main pathways: the classical pathway, the lectin pathway and the alternative pathway [33]. Antigen-antibody complexes on pathogen/allograft cell surfaces trigger the classical pathway, whereas the lectin pathway involves the interaction of mannan-binding lectin with microbial surfaces [33]. C3b, a product of the activated classical pathway, initiates the alternative pathway [33]. Complement is mainly produced in the liver but can be produced locally by many cell types at the site of inflammation [34]. In transplantation, complement mainly contributes to antibody-dependent mechanisms of rejection, and can cause direct damage via the release of activation products such as C3b, C5b-9, C3a and C5a [35,36]. IgG or IgM bound on the allograft endothelium can activate complement through their

interaction with C1q [4]. C1q fixation by IgG or IgM induces conformational changes in C1q that subsequently allows C1r cleavage and the activation of C1s (C1q, C1r and C1s are C1 components) [4]. C1r acts as an enzyme that activates C2 and C4 [4]. Activated C4 breaks into two fragments C4a (small fragment) and C4b (large fragment) [4]. Next, C4b forms a complex with C2a (the classical pathway C3 convertase) [4]. This complex in turn activates C3 to cleave into C3a and C3b [4]. At this point, C3b joins the C4b-C2a complex and forms a complex that cleaves C5 into C5a and C5b [4]. Finally, C5b induces the formation of the membrane-attack complex (MAC) [4]. MAC is composed of C5b-C6-C7-C8-C9 (C5b-9) and binds to the cell membrane causing cell lysis [4]. Complement fixation contributes mainly in the pathogenesis of acute and hyper-acute rejection [37].

Adaptive immunity

A) CD4 T cells

$CD4^+$ T cells, once activated, differentiate into T helper (Th) 1, Th2, Th17 or regulatory T cells (Tregs) [38]. $CD4^+$ T cell subsets are characterized by the cytokines they produce and the transcription factors they express [39].

Th1 & Th2

Th1 $CD4^+$ T cells are characterized by the production of TNF-α, IFN-γ and IL-2 and the expression of T-bet [40]. Allograft rejection mediated via Th1 $CD4^+$ T cell responses is mainly by supporting cytotoxic T cell activation, and delayed type hypersensitivity reaction mediated by IFN-γ [41,42,43]. Whereas, Th2 $CD4^+$ T cells are defined by the production of IL-4, IL-5, IL-6, IL-9 and IL-13

[40]; through IL-4 and IL-5 they recruit eosinophils leading to allograft rejection [44,45].

Th17 & Tregs

Th17 $CD4^+$ T cells are characterized by the production of IL-17-A and IL-17-F, and the expression of retinoic acid-related orphan receptor-α (RORα) and retinoic acid-related orphan receptor γT (RORγT)[46,40]. Rejection mediated by IL-17 is likely to be through the ability of this cytokine to rapidly recruit myeloid cells [47].

Tregs produce IL-10 and TGF-β and they express Forkhead box P3 (FOXP3) [48]. Tregs have the capacity to suppress exacerbated immune responses and maintain tolerance in autoimmune diseases and transplantation [49,50]. In addition, through cell-contact Tregs can dampen effector T cell responses [48]. However, Tregs can induce Th17 T cell differentiation and augment IL-17 production and can be themselves converted into Th17 T cells [51].

B) CD8 T cells

$CD8^+$ T cells, once activated in an antigen specific manner, can damage the graft via one of two pathways: granule exocytosis, or the Fas pathway [46].

C) B cells

Initially Szenbreg and Warner suggested that B cells were neither necessary nor sufficient for immune responses against an allograft [52]. They compared skin graft rejection in two groups of chickens. The first group was thymectomized, while the second group had their bursa of fabricius (B cell source) removed. The thymectomized group in this experiment accepted the

graft fully and permanently, while the B cell deficient group rejected the graft at the same time as the control [52]. These results suggested that rejecting a transplant was a process associated with the thymus, not the bursa of fabricius [52,53]. However, subsequent studies in this area have shown that B cells play a key role in the immune response to organ transplantation both in terms of rejection and tolerance, Zarkhin *et al.* showed that B cells amplify T cell responses during graft rejection [54]. In this study, they found that $CD20^+$ B cells in the graft are highly activated and express high levels of MHC II, supporting their hypothesis that B cells exacerbate anti-graft responses [54]. The presence of activated B cells in the graft was associated with reduced graft survival and a resistance to immunosuppressive agents [54]. Recently, the role of B cells was studied in chronic renal rejection, acute cardiac rejection, and skin rejection by depleting B cells from mouse transplant recipients using CD20 and CD19 depleting mAbs (CD20 mAb depletes mature B cells, while CD19 mAb deplete mature B cells, plasmablasts, and some plasma cells) [320]. Although depleting B cells has stopped the development of specific allo-IgG, it did not influence the rejection process of cardiac allografts [320]. In renal rejection, CD19 mAb treatment, but not CD20 mAb, significantly decreased the rejection and prevented allo-specific-IgG development [320]. However, depleting B cells in skin model accelerated the skin allograft rejection [320]. These results indicate that B cells can both up-regulate and down-regulate allo-immune responses depending on the intensity of the allo-responses and the organ transplanted [320].

In animal models, AbuAttieh *et al.* found that B cell deficient $JH^{-/-}$ mice rejected fully miss-matched skin graft at the same time as the controls [57]. On the other hand, it was shown by Waswska *et al*, in a cardiac transplant model, that only 85% of μMT mice rejected their grafts after 14 days, while 100% of WT mice rejected theirs [58]. In addition, they found that cardiac allograft rejection in μMT mice could be restored through donor-specific antibody administration [58]. The differences in the results obtained by the two groups might be due to the fact that μMT mice retain some B cells and antibody, while $JH^{-/-}$ mice completely lack B cells and antibodies [57].

In humans, depleting B cells by using anti-human CD20 mAbs has improved graft survival in some studies [59]. However, the mechanism by which depleting B cells impacts graft survival is not clearly understood. It could be a result of the inhibition of B cell antibody-dependent or antibody-independent responses. Some studies however suggest that depleting B cells is not beneficial to graft survival. For instance, Clatworthy *et al.* found that cellular rejection in kidney transplant was observed in 83% of anti-CD20 mAb treated transplant recipients, while it was only observed in 14% of anti-CD25 treated patients, indicating that B cells might regulate the immune system [60]. They found elevated serum concentrations of IL-10, IL-6 and TNF-α in the grafts of patients who had received anti-CD20 mAb compared to controls [60]. Clatworthy and colleagues suggested that the absence of B cells resulted in an increase in pro-inflammatory cytokines and activated antigen presenting cells and T cells [60]. The role of regulatory B cells has not been fully described in transplantation; however, it has been explored in autoimmune models and will be describe

latter. Altogether the results of Clatworthy *et al*. suggest that depleting B cells could lead to the depletion of regulatory B cell subsets and the deterioration of the graft [61].

A recent study by Newell *et al*. analyzed gene expression by peripheral blood lymphocytes isolated from immunosuppressant free spontaneously tolerant kidney transplant recipients and compared them to transplant recipients receiving immunosuppressive agents with stable renal graft function [55]. The group of tolerant recipients who were not receiving immunosuppressive drugs expressed higher levels of mRNA for IGKV4-1, IGLL21 and IGKV1D-13 [55]. These genes encode the variable region segments of Ig light chains [55]. Therefore, Newell and colleagues called it a “B-cell signature” and suggested that these genes could help in distinguishing those kidney transplant recipients who need immunosuppressive agents from those who do not [55, 56].

These mixed results from the literature suggest that B cells can exhibit both pathogenic as well as protective roles in transplantation, but this answer is not completely clear yet [66].

Antibody

Antibody can be involved in the three types of rejection: hyper-acute, acute and chronic rejection [63]. Antibodies mediate rejection by two mechanisms: complement-dependent and complement-independent pathways [63]. In the complement dependent pathway, the classical complement pathway is activated resulting in membrane attack complex formation that leads to cellular injury [63]. For example, C1q binds to IgM and IgG that have attached to alloantigen on the allograft epithelium, and this initiates the classical

complement pathway [63]. Moreover, the complement products (C3a & C5a) act as chemo-attractants for inflammatory cells whose infiltration results in inflammation [63]. In the complement independent pathway innate immune cells such as Natural Killer (NK) cells and macrophages are involved through their interaction with antibody bound to the allograft via their cell surface Fc receptors [63]. These interactions will lead to antibody dependent cell cytotoxicity (ADCC) [63].

1.1.4 Transplant tolerance

Tolerance is the state defined as the acceptance of the allograft by the recipient without the need for immunosuppressive drugs [2]. Although globally immunosuppressive treatments help to increase the degree of success of transplantation they can have many side effects such as infections and malignancies [64]. As a result, attempts have been made to replace the use of globally immunosuppressive drugs with more targeted therapies. One example is the use of Rituximab for B cell depletion. Rituximab is a humanized anti-CD20 antibody that depletes B cells [65]. However, as mentioned earlier, the depletion of B cells has provided mixed results, suggesting that B cells can play both pathogenic as well as protective role in transplantation [66]. Another approach for the induction of tolerance to allografts has been to achieve mixed hematopoietic chimerism [1]. This constitutes the depletion of the hematopoietic system and its reconstitution with syngeneic as well as allogeneic bone marrow [1]. This procedure however is associated with unpleasant side effects [1]. Other approaches for the induction of transplant tolerance include the use of

antibody-mediated blockade of co-stimulatory molecules, or adoptive cell therapy with *ex vivo* expanded Tregs or DCs [1].

One of the barriers to tolerance induction that face scientists is the presence of memory T and B cells [1,2]. Memory T cells respond faster than naïve T cells to antigens with little or no requirement for high antigen dose or co-stimulation, and for that reason memory T cells are considered one of the greatest challenges to the induction of tolerance [1,2]. Memory T cells that are specific for alloantigens can be generated in three different ways [1,2]. One-way is through pre-exposure to antigens via previous transplantation, pregnancy or prior blood transfusion [1,2]. Secondly, T cells can cross-react with and recognize more than one antigen [1,2]. This cross reactivity might be one of the reasons behind the complexity of generating tolerance in primates and humans, making it necessary to keep animals in pathogen free housing to study transplant immunology [2,67]. Finally, memory T cells can be generated as the result of homeostatic proliferation in which T cells under self antigen-MHC complex; cytokines and chemokines expand in a lymphopenic environment [1,2]. Memory B cells, exist as a result of previous sensitization [1,2], and get rapidly activated in response to their re-exposure to immunizing antigen [68,69,70]. Moreover, they require less help from T cells and lower antigen dose than naïve B cells [71]. Memory B cells are associated with complement fixation through IgG causing hyper-acute rejection [1].

1.1.5 The role of Major Histocompatibility Complex (MHC) molecules

The main cause of allograft rejection is the capacity of T cells to recognize alloantigen, which is mainly encoded within the MHC genes [1]. MHC genes in humans are known as human leukocyte antigens (HLA), and they are divide into MHC I and MHC II genes [64]. HLA-A, HLA-B and HLA-C are the three genes that encode MHC I molecules, while MHC II genes are composed of HLA-DQ, HLA-DP and HLA-DR [64]. In mice, MHC molecules are encoded by the H-2 region, which includes class I and class II molecules [64]. Class I includes three encoding genes K, D and L, and for class II the encoding genes are I-A and I-E [64].

1.2 B cells

1.2.1 B cells development

In general, B cells are divided into two distinct populations: B-1 and B-2 B cells. MZ and FO B cells represent the mature B2 populations [72]. B1 B cells can be subdivided into B1a and B1b B cells according to their CD5 expression (B1a are $CD5^+$ while B1b are $CD5^-$) [72]. B1 B cells are mainly found in the peritoneal and pleural cavities, and produce natural antibodies, in particular IgM and IgA [72]. Natural antibodies are polyreactive antibodies with low affinities that have specificities for a wide variety of antigens such as lipids, nucleotides and polysaccharides [73,74,75]. Unlike B2 B cells, B1 B cells are activated via IL-5, IL-10, TLRs or whole bacteria, and not via their BCR [76,77]. Activated B1 cells migrate into the omentum, spleen, regional lymph nodes or intestinal lamina propria [76,78]. Subsequently, activated B1 cells differentiate and produce natural IgM and IgA [76,78]. Therefore, B1 cells are innate-like B cells [79]. Although it is still unclear how B1 cells develop, two hypotheses have been proposed [80,81]. First of all, the “lineage hypothesis” which suggests that B1 and B2 arise from different B cells precursors [80,81]. Secondly, the “induced differentiation hypothesis” which suggests that B1 and B2 B cells develop from a common precursor; depending on the specific signals that the precursor receives during maturation and selection cells either differentiate into B1 or B2 B cells [80,81].

B2 cells develop throughout adult life in the bone marrow (BM) [3,82]. The medullary cavities in bone are occupied by a soft tissue known as the BM, which is surrounded by an outer structure called endosteum that is lined by

variety of cells such as stromal reticular cells [3,82]. The latter cells are also found close to the central sinus, where they are called adventitial reticular cells [3]. The BM is rich with medullary vascular sinuses that drain into the central sinus where blood is collected and exits the BM [83]. The adventitial reticular cells serve as a gateway for B cells to enter the central sinus, and the circulation [3].

Prior to the release of B cells into the circulation, a process of selection and programmed cell death take place. Stromal reticular cells play a key role in the development of B cells and survival [3]. B cells progenitors interact with stromal reticular cells, and are promoted to proliferate and mature [3]. The binding of B cells progenitors and early pre-B cells to stromal cells is mediated through the interaction between VCAM-1 on stromal cells and integrin VLA-4 on developing B cells, also it involves the interaction between other cell adhesion molecules [98]. CD117 on pro-B cells binds to SCF (stem-cell factor) expressed on the surface of the stromal cells, this interaction induces B cell progenitor proliferation via the activation of kinase (phosphatidylinositol (PI)–3 kinase/protein kinase (PI-3 kinase)) [98]. IL-7 is produced by stromal cells and it is required at early stages of B cell development where it is essential for cell proliferation and development [98]. The stromal cells also continuously produce the chemokine CXCL12 (stromal cell-derived factor 1, SDF-1) that retains the cells in the stroma during their development in the BM [98].

At the earliest stage of B cells maturation upon their interaction with stromal cells, B cells are known as pro-B cells [98]. At this stage, immunoglobulin (Ig) heavy chain locus rearrangement occurs. Immunoglobulins are composed of

heavy and light chains and both contain variable (V) and constant (C) regions [98]. The V domain of the light chain is encoded by two DNA segments the V_L gene segment and the J_L (Joining) gene segment, whereas the heavy chain, the V region is encoded by V_H and J_H gene segments together with a third segment known as diversity or D_H gene segment [98]. The C region is encoded in different segments and joins the V domain at the end by RNA splicing [98]. As mentioned above, Ig heavy chain locus rearrangement takes place first, in which D_H join J_H segment (early pro-B cell) then DJ_H joins V_H (late pro-B cell) [98]. Subsequently, the μ heavy chain (part of IgM) is expressed intracellularly as a result of VDJ_H productive joining, this stage known as late pro-B cell stage [98]. At this stage, the μ chain combines with a surrogate light chain. The joining of μ chain to the surrogate light chain leads to the expression of the pre-B cell receptor on the cell surface that provide signals essential to stop VDJ_H rearrangement and to promote cell division into small pre-B cells [98]. Here, light chain rearrangements begin.

The light chain has two types: Lambda (λ) and Kappa (κ). Immunoglobulin consists of either κ or λ never both, however no functional differences have been found between them [98]. The light chain rearrangement involves the joining of V_L to J_L [98]. In general, κ chain locus rearrangement occurs before λ; when κ rearrangement fails λ rearrangement takes place, and when λ rearrangement fails the cell die [98]. Pre-B cells that express self-reactive immunoglobulin or have rearranged non-productive receptor genes undergo apoptosis and are phagocytosed by BM resident macrophages [3]. Surviving cells differentiate further into immature B cells before they leave the BM and

interact with adventitial reticular cells that facilitate their exit into the circulation as transitional-1 (T1) B cells (a transient transitional B cells stage), which express IgM, low levels of CD21, but not IgD or CD23 [3,84]. Immature B cells undergo a process of receptor editing and programmed death (negative selection) [98]. Immature B cells that recognize self-antigens in the BM are retained and can rearrange a new light chain that combine, a process called receptor editing [98]. However, those cells that form new receptor that again recognizes self-antigen undergo programmed death [98]. Successful receptor editing, in which the new receptor is not reactive, leads to survival [98].

T1 B cells have the potential to recirculate between the BM and the spleen [84]. In the splenic follicles T1 B cells mature into transitional-2 (T2) B cells that express IgD and CD23 [84]. At this stage, T2 differentiate into either follicular (FO) B cells or into marginal zone precursor (MZP) B cells that in turn mature into marginal zone (MZ) B cells [84]. The transitional stage of B cell development has also been suggested to contain an additional transitional group termed transitional-3 (T3) [85]. T3 cells were proposed by Allman *et al.* who suggested that T3 give arise to FO B cells without the proliferative burst previously ascribed to T2 cells, which give arise to both FO and MZ B cells [85]. However, this hypothesis has been challenged and it has been suggested that the T3 population comprises anergic B cells rather than the progenitors of FO B cells [86]. Moreover, Pillai *et al.* have suggested that T2 exist in two populations: T2 Follicular progenitors (T2-FP) and T2 Marginal Zone progenitor (T2-MZP) [87,88]. T2-FPs have been suggested to be present in all secondary lymphoid organs and differentiate into FO B cells [85,89], whereas T2-MZPs are

restricted to the spleen and differentiate into MZ B cells [89,90]. Whether T2 B cells differentiate into FO (IgD^+, IgM^+, $CD23^+$ and $CD21^{mid}$) or MZP and MZ B cells depends on several factors such as the strength of BCR (B cell antigen receptor) signals; it also requires NOTCH 2 and canonical Nuclear Factor-κB (NF-κB) activity [84].

MZP B cells express CD23, CD1d, CD21, IgM and IgD and mature into MZ B cells that express IgD, IgM, CD1d and CD21, but are $CD23^-$ [84]. MZ B cells are located between the marginal sinus and the red pulp [84]. As mentioned earlier, MZ B cells express high levels of CD1d and CD21 [84]. CD1d expression by MZ B cells facilitates lipid and glycolipid presentation to invariant natural killer T (iNKT) cells, while the expression of CD21 allows MZ B cells to transport circulating immune complexes to the follicles in the spleen and deposit them with follicular dendritic cells [91,92,93,94]. MZ B cells share some features with memory B cells such as the ability to self-renew, a long-life span and a pre-activated phenotype [95,96,97].

1.2.2 Germinal Centre formation

Following exposure to microbial antigens B cells become activated by the internalization of small soluble antigens that gain access to the follicle via specialized transport system; these antigens are recognized by the B cell surface IgM and IgD receptors [99,100,101,102]. However, B cells require antigen sampling macrophages and DCs (located in the marginal zone area (spleen) or paraconrtical area (lymph nodes)) to present large antigens [99,100,101,102]. Following this activation of B cells through their surface Ig receptors they migrate to the T-B border area (the border between T zone and follicle) [103]. At T-B border, B cells meet and make cognate interactions with specialized $CD4^+$ T cells, known as T follicular helper (T_{FH}) cells [104,105,106,107,108]. The T_{FH} cells are generated following the activation of $CD4^+$ T cells by IL-12 and via MHC II expressed by activated DCs that have migrated from the invasion site to T cell zone [103,109]. Here, the initial activation of B cells through IL-4, IL-21 and IFN-γ produced by T_{FH} cells and their interaction with CD40L on T_{FH} cells, initiates the development of antibody-secreting cells (ASC) [103,110]. ASC develop extra-follicularly, and produce a fast antibody response to antigen, either in response to T cell independent type 2 (T1-2) antigens, which are large antigens that express epitopes in highly repetitive manner and they can activate B cells directly via their BCR without the need of co-stimulatory signals [111] (in case of MZ & B1 B cells [112]), or in a T cell dependent manner [113]. Extra-follicular ASC undergo apoptosis around a week after infection/immunization, and they mainly produce non-somatically mutated IgM [113].

Both MZ and FO B cells can form extra-follicular ASCs, however, after a week activated FO B cells start developing a Germinal Centre (GC) in the follicle [3]. Rapid proliferation of the activated FO B cells excludes the non-activated IgM^+ and IgD^+ B cells to the surroundings of the GC to form a structure known as the mantle zone [3]. The follicle at this point is known as the secondary follicle [3]. The secondary follicles consist of a dark zone, where B cells undergo clonal expansion and somatic hyper-mutation, and a light zone, where B cells selection and class switching occurs [3].

Clonally expanded B cells are known as centroblasts that subsequently undergo somatic hyper-mutation [3]. The latter is a process in which the variable region of the rearranged Ig gene is modified in order to generate a BCR with a greater affinity for the targeted antigen [110,114,3]. Modified centroblasts are termed centrocytes, which enter the light zone and undergo selection by the follicular DCs [3]. Only B cells with high affinity get to survive and interact with T_{FH} cells in the follicles where they may class switch their Ig heavy chain constant region gene from IgM and IgD to other classes of Ig [3,103]. B cells that fail to pass the selection process apoptose and are phagocytosed by follicular macrophages [3]. Class switched B cells subsequently differentiate either into plasma or memory B cells [3,103,114]. The GC diminishes after this stage [114].

Signals involved in the commitment decision of B cells to become plasma or memory B cells are not fully known [115,116,117]. However, several transcription factors have been identified to be involved in this decision, for example, paired box protein 5 (PAXS) is important for memory B cell

differentiation, while the repression of PAXS together with the expression of X-box-binding protein 1 (XBPI) is crucial for plasma cells commitment [115,116,117]. In mice, GC B cells are characterized by the high expression of GL7 (T and B Cell Activation Marker) and CD95 and they are $CD38^-$, whereas plasma and memory B cells are negative for both markers (GL7 & CD95) [118]. However, plasma cells express high levels of CD138, and memory B cells are $CD138^-$, $CD95^-$ and $CD38^+$ [118]. Memory B cells generate IgG expressing extra-follicular areas as well as IgM memory B cell follicles in the lymph nodes [119]. Upon secondary antigen exposure, IgM memory B cells induce a secondary germinal centre reaction, while IgG memory B cells rapidly differentiate into plasmablasts [119,120]. Memory B cell expansion and differentiation is controlled by their interaction with antigen specific memory T_{FH} cells residing in the draining lymph nodes to induce their responses upon antigenic re-challenging. In addition non-specific stimulation of memory B cells through their TLRs appears to be important for the expansion of polyclonal memory B cells [103,321,121]. Although the memory B cell responses induced by T_{FH} cells in draining lymph nodes occur more rapidly than memory B cells responses induced in memory secondary lymphoid organs devoid of T_{FH} cells, these responses are still kinetically slow, and the generation of faster responses requires their interaction with innate cells such as invariant NKT (iNKT) cells, DCs, macrophages and other cells [103,321]. In fact, B cells produce early waves of IgM and IgG when they interact with T_{FH} cells in the presence of the innate immune cells [103]. On the other hand, plasma cells migrate to the BM, and continuously produce antibodies [122,123,124].

1.2.3 B cell functions

In addition to the role of B cells in the humoral arm of the immune system, where they produce antibodies for opsonization and complement activation, initiate cellular cytotoxicity, form immune complexes and facilitate endocytosis, they also participate in shaping the immune system responses in a variety of antibody-independent ways [125]. For example, B cells can be very potent antigen presenting cells (APC) that capture specific antigen via their BCRs and present antigenic peptides in an antigen specific manner via their MHC class I and MHC class II to both CD8 and CD4 T cells respectively [126,127,128]. Activated B cells internalize antigens through their BCR, process and present it by MHC class I and MHC class II [125,126]. In order for B cells to function as APCs they need to receive two signals, the first upon encountering their specific antigen (BCR signal), and the second through their interaction with CD40L on activated $CD4^+$ T cells [133]. B cells can function as APCs for CD4 T cells without the CD4 T cells having been pre-activated by other APCs such as DCs [133]. Here, B cells internalize and process antigens and present them on MHC II that interact with TCRs expressed on CD4 T cells, and express CD86 molecules that cross-link with CD28 on CD4 T cells [129,130,131,132]. These two signals are sufficient for the expression of CD40L on CD4 T cells that then interacts with CD40 on B cells. Following CD40-CD40L interaction CD86 expression is maintained and CD80 and MHC II are up regulated, and provide full activation for both T and B cells [129,130,131,132,133].

As well as the ability of B cells to provide signals to T cells in an antigen-dependent manner, they deliver essential signals for the activation and

proliferation of both naïve and memory CD4 T cells through CD80 and CD86 [134,135,136]. An experiment that was performed using mice that lacked the expression of CD80 and CD86 on their B cells demonstrated that the expression of CD80 and CD86 by B cells was needed for auto-reactive T cell proliferation and activation in a model of arthritis (proteoglycan (PG)-induced arthritis) [136]. *In vitro,* challenging CD4 T cells obtained from arthritic mice whose B cells were CD80/CD86 deficient with PG (obtain from cartilage) resulted in less proliferation of CD4 T cells compared to the WT CD4 T cells [136].

B cells produce a variety of pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ and IL-12 [125]. According to their cytokine profiles B cells have been divided into B effector 1 ("Be1") and "Be2" cells [137,138,139]. The presence of antigen, Th1 T cells or/and TLRs signals as well as Th1 cytokines (IFN-γ and IL-12) leads to the generation of Be1 B cells that secret IL-4, IL-13, IL-2, IL-10, IL-6 and TNF-α [137,138]. On the other hand, Be2 cells produce IL-2, IL-4, IL-13, TNF-α, IL-6, IL-10 and small amount of IFN-γ and IL-12 in response to T cell priming, antigen and Th2 cytokines [137,139]. Together with the capacity of B cells to support T cell inflammatory immune responses they also can act as regulatory cells through several well-defined mechanisms including T cell deletion, the induction of anergy and cytokine mediated suppression [140,141,142].

1.3 Regulatory B cells

The history of regulatory B (Bregs) cells starts in the1970s, with the idea that B cells down-regulate the immune system through the production of “inhibitory” antibodies [143]. This study was later followed by a series of papers emphasizing that B cells have antibody-independent immunoregulatory function [144]. For instance, in 2002, Mizoguchi and colleagues found that IL-10 producing CD1d high B cells have the capacity to down-regulate chronic colitis [145]. In 2007, regulatory B cells were phenotyped in more detail by Evans *et al.* in an arthritis model, where they described Bregs that express high levels of CD21, CD23 and IgM the characteristic markers for T2-MZP B cells [146]. However, various phenotypically distinct subsets of B cells with regulatory functions have been subsequently identified in models of inflammation and autoimmunity. Generally, these cells are characterized by IL-10 production. In this section I will discuss the different subsets of B cells that have been suggested to be regulatory and identify their regulatory role in different models.

1.3.1 Phenotypically distinct subsets of regulatory B cells

The two most well defined subsets of B cells with regulatory function are the T2-MZP B cells, which will be termed “Bregs”, and the B10 [144]. These two subsets share some common features such as CD19, CD21, CD24 and CD1d expression (Figure 1.1) as well as being mainly localized in the spleen, and they both produce IL-10 [144]. However, the signature of B10 B cells is their expression of CD5 that Bregs do not express, while Bregs express CD23 that is not found on B10 B cells (Figure 1.1) [144]. Also, Bregs suppressive capacity was initially linked to CD40 engagement, while B10 was associated with TLRs ligands (in particular LPS) [144].

MZ B cells ($CD21^+$, $CD24^+$, $CD23^-$ and $CD5^-$) have also been identified to be regulatory (Figure 1.1), however, the idea of MZ B cells being regulatory is controversial. In a contact hypersensitivity (CHS) model the adoptive transfer of MZ B cells down-regulate CHS responses in $CD19^{-/-}$ mice [147], whereas their adoptive transfer fails to protect from the disease in CIA (Collagen-induced arthritis) model [144]. Recently, an additional subset has been added to the list, the B killer cell, called killer because they induce the apoptosis of arthritogenic T cells in an arthritis model [144]. B killer cells induce apoptosis via expression of Fas ligand (Figure 1.1), and they share CD5 expression with B10 B cells [144]. Additionally, stimulating B cells with a GM-CSF and IL-15 ‘fusokine’ (GIFT15) induces a subset of regulatory B cells that express T2-MZP B cells markers as well as CD138 (a plasma cell marker). When adoptively transferred GIFT15 induced Bregs can suppress experimental autoimmune encephalomyelitis

(EAE) through IL-10 production, up-regulation of MHC II and STAT-6 expression [148].

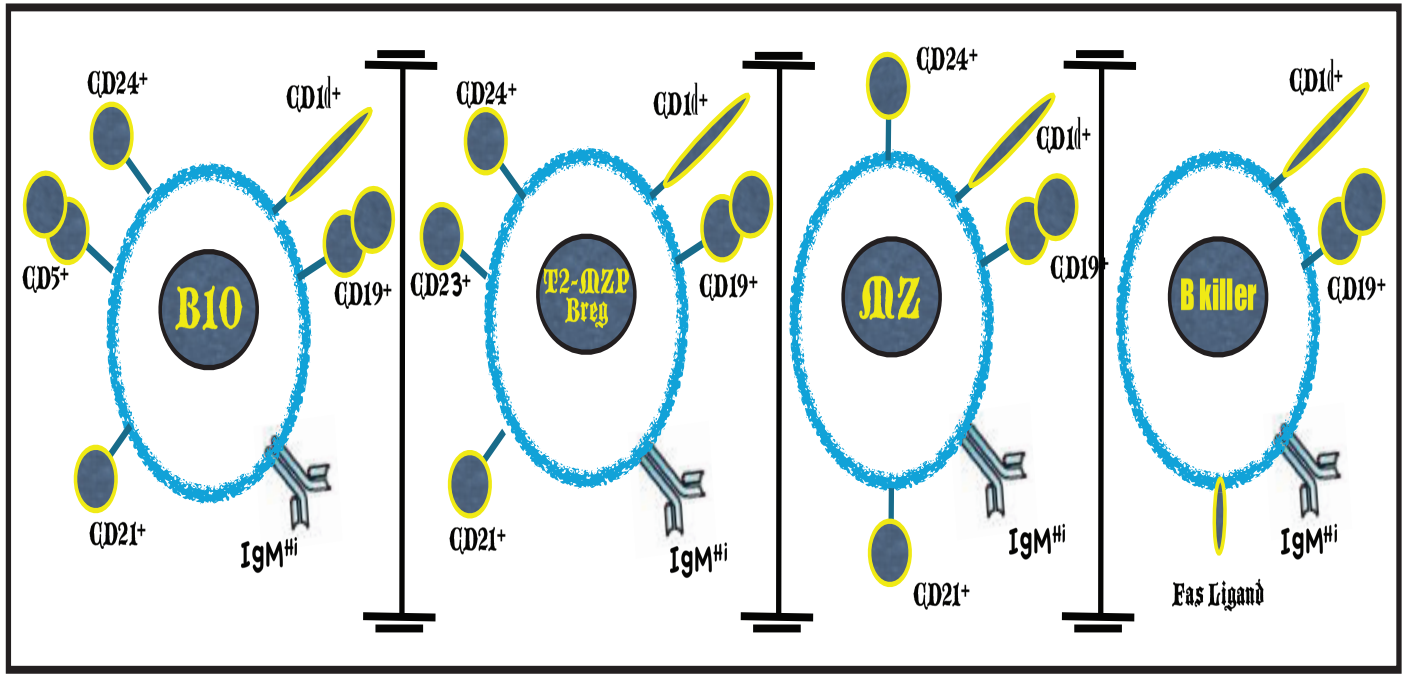

Figure 1.1 phenotypically distinct subsets of regulatory B cells. B10 and T2-MZP B cell subsets share some common features such as CD19, CD21, CD24 and CD1d expression. However, the signature of B10 B cells is their expression of CD5 that Bregs do not express, while Bregs express CD23 that is not found on B10 B cells. T2-MZP and MZ B cell subsets share as well some common features such as CD19, CD21, CD24 and CD1d expression, however, MZ B cells do not express CD23. B killer cells express Fas ligand [144].

A) T2-MZP B cells "Bregs"

Bregs with a T2-MZP B cell phenotype have been reported in several autoimmune models such as CIA and a murine model of lupus [146,149]. In arthritis, the transfer of T2-MZP B cells isolated from naïve mice suppresses the development of CIA in DBA/1 mice [146]. Moreover, the transfer of T2-MZP B cells obtained from mice in the remission phase of arthritis has a more powerful regulatory effect [146]. Adoptive transfer of low numbers (4×10^5 cells) of T2-MZP B cells from mice in remission from arthritis achieved a greater degree of suppression of arthritis than transfer of the same number of T2-MZP B cells from naïve mice [146].

The mechanism described to be associated with the suppressive capacity of T2-MZP B cells in the CIA model was the ability of T2-MZP B cells to produce IL-10 [146]. It was observed that the majority of IL-10 produced by splenic B cells isolated from mice in the remission phase of arthritis was by T2-MZP B cells following re-stimulation with collagen *in vitro* [146]. Moreover, the adoptive transfer of IL-$10^{-/-}$ T2-MZP B cells did not protect the mice form CIA [146].

In MRL/lpr mouse model of lupus, the absolute number of T2-MZP B cells correlated with the severity of the disease, however, the adoptive transfer of T2-MZP B cells failed to protect from the disease [149]. T2-MZP B cells, in this model regain their suppressive capacity after being activated *in vitro* with anti-CD40 mAb, following which the transfer of these cells delayed the onset of lupus like disease and prolonged the survival of the mice [149].

B) Marginal Zone B cells

MZ B cells produce IL-10 and some IL-6 in response to TLR-2 and 4 ligation, a feature that is amplified when they are co-stimulated via CD40 [150,151]. IL-10 producing MZ B cells were able to delay colitis in a $G\alpha i2^{-/-}$ mouse model mouse of colitis [152]. The transfer of T cells obtained from $G\alpha i2^{-/-}$ mice to syngeneic $RAG2^{-/-}$ mice induces colitis. However, transferring the T cells together with WT splenic MZ B cells induced temporary protection [152]. This protection was not however as efficient as that observed using mesenteric lymph node B cells which provided permanent protection from colitis [152]. Notably the purity of MZ B cells in these experiments were around 55%, suggesting that this suppression could be due to the presence of other B cell subsets, including T2-MZP B cells [152]. In the CIA arthritis model, adoptive transfer of B cells that had been isolated from arthritic mice treated with apoptotic cells could protect mice from arthritis. IL-10 was shown to be necessary for this protective effect and, given that MZ B cells were the major IL-10 producers *in vitro*, it was suggested that MZ B cells were the regulatory subset [290]. However, Evans *et al.* demonstrated that the transfer of purified MZ B cells failed to protect from the disease in the CIA model [146]. Therefore, as mentioned earlier, MZ B cells suppressive capacity is a controversial area.

C) B10

Tedder and colleagues were the first to identify B10 cells. In a contact hypersensitivity model (CHS) they found that $CD19^{+}$ $CD5^{+}$ $CD1d^{+}$ IL-10 producing B10 B cells were markedly increased in hCD19Tg mice (mice which overexpress CD19) [153]. These mice displayed reduced oxazalone mediated inflammation compared with WT mice [153]. In contrast, in $CD19^{-/-}$ mice the onset of inflammation was more severe compared to WT mice, however, the transfer of B10 B cells delayed the onset of disease via an IL-10 dependent mechanism [153]. In an EAE model, a Th1 dependent model of human multiple sclerosis, [154,155] the depletion of B cells using a CD20 mAb 7 days prior to EAE induction exacerbated the disease [154,155]. The disease was normalized upon the adoptive transfer of $CD5^{+}$ $CD1d^{+}$ B cells, but not other B cell subsets. The adoptive transfer of IL-$10^{-/-}$ B10 B cells did not delay EAE development, supporting the hypothesis that the suppressive capacity of B10 B cells is mediated via IL-10 [156]. The link between T2-MZP Bregs and B10 B cells is still under investigation [144]. However, it has been suggested that B10 and T2-MZP could be either distinct subsets or that T2-MZP could be the precursors of B10 [144].

D) FasL$^+$ "Killer" B cells

As discussed earlier "killer" B cells express Fas ligand, which induces the death of target cells through direct contact with Fas [144]. These B cells share CD5 expression with B10 cells [144]. The expression of Fas ligand by B cells was first reported after activating human B cells with LPS or PMA/ionomycin [157], followed by a series of studies addressing the pathogenic affect of FasL expressing B cells on the progress of several malignant B cell diseases and during viral infection [158-164]. However, in a schistosoma infection model, CD5$^+$ FasL$^+$ B cells were found to be important in inducing the apoptosis of antigen-specific T cells [165]. Moreover, they can induce the apoptosis of arthritogenic T cells leading to the suppression of arthritis [165,166]. The tolerogenic properties of "Killer" B cells were also addressed in a transplant model (male to female skin graft) [167]. In this model, the adoptive transfer of WT splenic B cells was able to induce tolerance, whereas the transfer of FasL$^{-/-}$ B cells did not exhibit the same effect [167].

1.3.2 Regulatory B cell requirements and functions

IL-10 producing B cells require signals through TLRs, CD40, CD80, CD86 and BCR to achieve their optimal suppressive capacity. Signals via TLRs have been reported to be required for B cell regulatory function, in particular for B10 B cell function, in which stimulating B10 with LPS (TLR-4) *in vitro* induced IL-10 production that was essential for their suppression capacity [153]. Similarly providing B cells with strong stimulation through CD40 by using anti-CD40 mAb directs B cells to produce IL-10 as shown in several autoimmune models [168]. For instance, chimeric mice with $CD40^{-/-}$ B cells suffer from a more severe EAE than WT controls [168]. Furthermore, the adoptive transfer of *in vitro* anti-CD40 mAb activated B cells suppresses the development of arthritis [169]. The importance of BCR signals for B cells with regulatory function is still under investigation. However, severe EAE developed in $CD19^{-/-}$ mice (CD19 is part of the BCR signaling complex) compared to WT mice, suggesting that the BCR may participate in the function of B cells with regulatory function. Nevertheless, this area requires more study [153,170,171].

In addition, it has been documented that CD80 and CD86 are required for B cells to exert their regulatory function. In EAE, reconstitution of irradiated μMT mice with BM from $B7^{-/-}$ mice failed to protect mice from the disease after the adoptive transfer of encephalitogenic T cells. The mice in this experiment were protected only following reconstitution with BM from mice whose B cells expressed CD80 and CD86 [172].

The ability of B cells to exert suppression might require the combinations of all the stimuli mentioned above. The level of stimuli required by the B cells to

exhibit regulatory function and whether single or multi stimuli is required is a field that needs to be investigated.

1.3.3 Regulatory B cells and transplantation

There are as yet no mouse studies that link T2-MZP B cells or B10 B cells to the induction of transplant tolerance. A recent study, however, has identified TIM-1 (T cell Ig domain and mucin domain) as a marker for IL-10 producing B cells, and that TIM-1 plays an important functional role in IL-10 producing B cells [173]. Here, anti-TIM-1 was used to induce tolerance in an islet transplant model [173]. This was explained by an increase in TIM-1^+ B cells expressing IL-4 and IL-10 in anti-TIM-1 treated mice [173]. In contrast the treatment of B cell depleted mice with anti-TIM-1 accelerated islet allograft rejection [173]. Islet graft survival was enhanced by treating B cell depleted recipients with WT B cells together with anti-TIM-1 [173]. These results suggested that B cells play a role in TIM-1 induced tolerance [173]. It was also observed that anti-Tim-1 induced tolerance is associated with Th2 shift, a decrease in IFN-γ with an increase in IL-4 and IL-10 expression [173]. Treating islet recipient B cell depleted mice with anti-TIM-1 increased IFN-γ expression, suggesting that B cells play a role in the Th2 shift observed in this model [173]. In addition, the adoptive transfer of TIM-1^+ B cells, isolated from anti-TIM-1 treated recipient mice at day 14, to untreated islets recipient mice significantly prolonged the graft survival compared to adoptive transfer of TIM-$1^{-/-}$ B cells and to adoptive transfer of Tim-1^+ B cells isolated from naïve mice [173]. These results again support the idea that B cells with regulatory function need to be activated in order to exert their suppressive function [173].

1.4 Galectins

Galectins are carbohydrate-binding proteins that contain conserved carbohydrate recognition domains (CRD) [174]. CRD are used to classify galectins, and they are responsible for carbohydrate binding [175]. In mammals galectins are divided into three groups according to their CRD [176,177]. The protypical galectin group consists of one CRD and can form dimers [175]. One group that contain galectin-3 only because of their unique structure (chimegic galectins), and the tandem repeat group, which contain at least two CRD linked through small peptide (Figure 1.2) [175]. Saccharides and galactose can bind to galectin carbohydrate binding sites, and different galectins can accommodate saccharides bound to galactose differently [178,179]. Therefore, each galectin is specific for different carbohydrate oligosaccharides [178,179]. Galectins can be found both extracellularly and intracellularly [174]. Extracellularly, galectins are secreted from cells via non-classical secretory pathways. Unlike classical pathways in which the secreted proteins contain an N-terminus that is required to direct the development of the polypeptide and the ribosome of the endoplasmic reticulum (ER), the proteins secreted via the non-classical pathway such as Galectins do not contain the N-terminus and do not get secreted from the ER [180,181,322]. Galectins function as bridges linking cells to each other and cells to their extracellular matrixes [174]. Galectins can also cross-link cell surface glycoproteins, triggering cascades of signals that mediate apoptosis, mitosis and cell cycle progression [174]. On the intercellular level, galectins mainly interact with proteins, and are involved in essential cellular functions such as pre-mRNA splicing [182,183], cell growth, apoptosis and cell-

cycle progression (Figure 1.2), however, exactly how galectins are involved in these processes is not known [174].

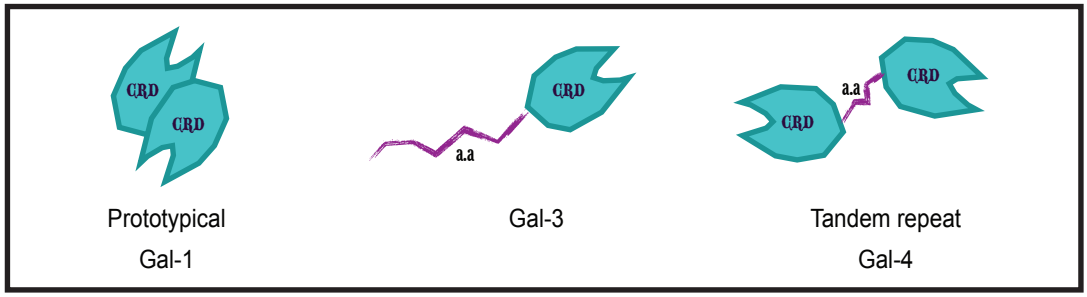

Figure 1.2 Galectin groups. 15 galectins have been identified and subdivided according to their CRD into 3 groups: prototypical group such as Gal-1, Gal-3 group, and tandem repeat group such as Gal-4 [175].

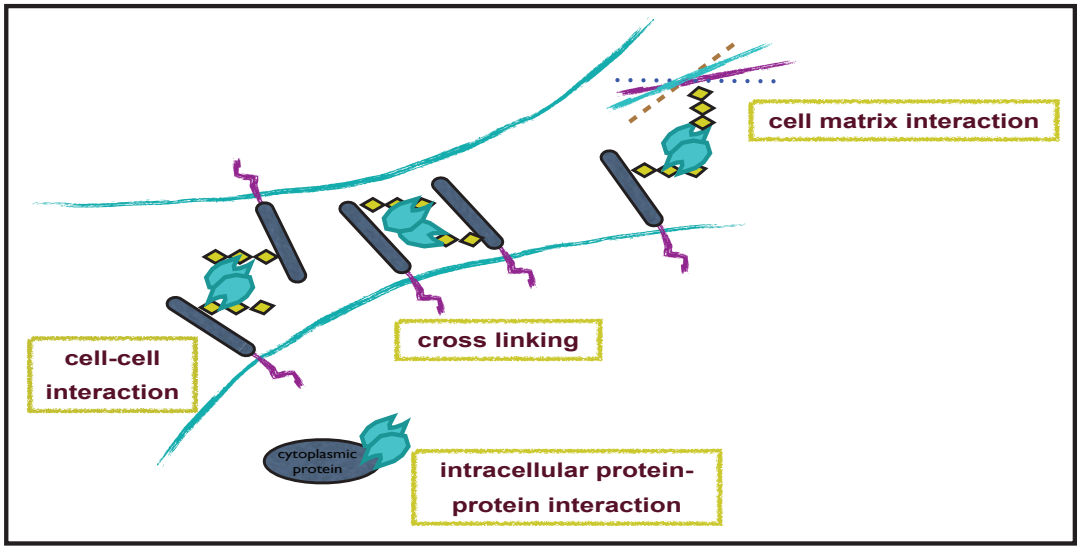

Figure 1.3 Galectin functions. Secreted galectins contributes in several extracellular functions such as facilitating cell-cell interaction, cross-linking cell surface receptors, and bridging cells to their extracellular matrixes. Intercellularly, galectins mainly interact with proteins, and are involved in essential cellular functions such as pre-mRNA splicing, cell growth, apoptosis and cell cycle progression [174,182,183].

1.4.1 Galectin-1

Galectin-1 (Gal-1) belongs to the prototypical group of galectins that contain one CRD [175] and exist as monomers and non-covalent homodimers. Each form has functionally different biological activities [184,185]. Homodimer or dimer Gal-1 (dGal-1) can separate into monomers (mGal-1) at low concentrations. These monomers can still bind to carbohydrate moieties, but with lower affinities [186].

Gal-1 is expressed both inside and outside the cells. On the intracellular level, Gal-1 can be found in the nuclei, cytosols and on the intracellular side of cell membranes [187]. In addition, Gal-1 binds to proteins intracellularly, while it binds to carbohydrates extracellularly [187]. Many extracellular carbohydrate ligands of Gal-1 have been identified that participate in Gal-1 mediated bridging of cells to each other, or to the extracellular matrix [187]. For instance, integrins and GM1 gangliosides are two of the major extracellular ligands for Gal-1. As mentioned above, Gal-1 binds to proteins intracellularly. Those proteins that have been identified as intracellular Gal-1 ligands do not however share structural similarities nor domains nor motifs, and the site of their interaction with Gal-1 is yet to be identified [188,189]. One example of a Gal-1 intracellular binding proteins is Ras. H-Ras-GTP recruits Gal-1 to the cell membrane forming stable complex (Gal-HRas) that in turn binds to Raf-1 and can activate the ERK pathway [188,189].

Gal-1 is involved in several cellular functions such as cell growth and cell migration. Gal-1 can regulate cell growth in a dose dependent manner. A Gal-1 low dose triggers cell mitosis, whereas a high dose inhibits proliferation

[190,191]. Gal-1 facilitates cell adhesion by bridging the cell surface integrins to their extracellular matrixes [192,193]. Gal-1 deficiency in glioma cells results in a decrease in their motility and adhesion [194,195]. In motility, increased expression of Gal-1 was associated with an increase in RhoA expression. RhoA is a protein involved in actin polymerization and depolymerization [194].

1.4.2 Gal-1 and T cells.

It has been reported that Gal-1 plays a critical role in T cell function; for example, Gal-1 can induce Th2 responses [175,203], influence T cell viability [202], T cell development [200,201,202], and many other functions. Also, Gal-1 has been identified as one of the functional markers for Tregs, as Gal-1 expression is up regulated following Treg activation through their TCR receptor [196]. Moreover, they found that blocking Gal-1 with anti-Gal-1 mAbs inhibited the suppressive function of the Tregs in humans [196].

In the context of T cell development cells undergo a process of positive and negative selection during their development in the thymus to ensure the generation of T cells with functional TCR [197]. The selection is based on the reactivity between T cells TCR and thymic APCs, which presents self-peptide (MHC) [198]. Gal-1 is expressed by splenic cells, lymph node and thymic APCs, as well as thymic endothelial cells [199,200,201]. It has been demonstrated that Gal-1 mediates the interaction between T cells and thymic endothelial cells [200,201], and induces apoptosis of $CD8^+$ $CD4^+$ and some $CD4^-$ $CD8^-$ thymocytes [200,201,202].

In addition to the involvement of Gal-1 in T cell development, Gal-1 can play an immunoregulatory role by shifting the immune responses from Th1 and Th17 to

Th2 responses [175]. Toscano *et al.* reported that Th1 and Th17 T cells, but not Th2 T cells, express a set of glycans on their cell surface that trigger cell death signals upon binding to Gal-1 [203]. Also, it has been reported that Gal-1 secreted by Th2 T cells induces selective apoptosis in Th1 T cells, whereas Gal-1 produced by Th1 T cells generates Th2 cytokine secretion [202]. Moreover, Gal-1 can induce IL-10 production in both $CD4^+$ and $CD8^+$ T cells [204].

1.4.3 Gal-1 and B cells

Different studies have shown that Gal-1 plays a key role in B cell function, in terms of their immunoglobulin production [205], development [206], and their influence on T cells [207].

A master regulator called Blimp-1 (B lymphocyte-induced maturation Protein-1) regulates the differentiation of plasma cells [205]. The ectopic expression of Blimp-1 was found to be associated with up-regulation in Gal-1 in mature B cells. Also, it was shown that deleting prdm1 (the Blimp-1 gene) resulted in a severe diminishing in the secretion of IgM, the expression of CD138, and Gal-1 secretion upon LPS stimulation *in vitro* [205]. These results suggest that plasma cell differentiation is associated with Gal-1 induction in a Blimp-1 dependent manner [205]. Furthermore, B cells that express a mutant Gal-1 that is retained within the cells had defective Ig production compare to WT B cells, suggesting that extracellular Gal-1 promotes Ig production by B cells [205].

In B cell development, Gal-1 is involved in anchoring the interaction between stromal cell integrins and the pre-BCR on B cells, providing survival signals during their development [208].

1.4.4 Gal-1 in autoimmunity

The role of Gal-1 has been studied in several autoimmune models including EAE [209], arthritis [210] and colitis [213]. The treatment of EAE mice with Gal-1 resulted in a delay in the development of the disease [209]. In arthritis, injecting DBA mice at day 1 following the induction of CIA with fibroblasts engineered (cells were transfected with mGal-1 DNA) to secrete Gal-1 abrogated the disease. In this study, they also found that the lymphocytes isolated from draining lymph nodes exhibited Th2 immune responses at the end of the treatment [210].

1.4.5 Gal-1 in transplantation

Xu *et al*. demonstrated that Gal-1 treatment 7 days prior to renal transplant prolonged the graft survival. These results were associated with a reduction in serum IFN-γ and $CD8^+$ T cells [211]. In addition, it has been reported that Gal-1 treatment improved the rate of morbidity and mortality in a model of GvHD by reducing Th1 responses and increasing the numbers of Treg cells [212]. As Gal-1 has the potential to suppress allo-reactive T cell responses it is possible that Gal-1 could be used as immunosuppressive agent in transplantation [187].

1.5 Toll-like Receptors

For pathogens to successfully invade our bodies they have to evade two lines of defence: the innate and adaptive immune systems [214]. Innate immunity represents the first line of defence and contributes to the priming of the adaptive immune response [214]. Pathogen components are sensed by pattern-recognition receptors (PRRs) that are expressed on innate immune cells [214]. These molecules recognize pathogen associated molecular patterns (PAMP) [214]. PRRs can also recognise damage associated molecule patterns (DAMP), which are revealed following cell necrosis [215,216].

TLRs are member of PRR family [217]. So far 10 TLRs have been identified in humans, while in mice 12 TLRs have been listed [217]. The engagement of TLRs activates several signaling pathways that lead to NF-κB (Nuclear Factor-κB) and IRFs (Interferon-regulatory Factors) translocation, and the subsequent release of pro-inflammatory cytokines (associated with NF-κB) and type I interferons (associate with IRFs) [217].

1.5.1 TLRs ligands and signaling

TLRs recognize PAMPS on both extracellular and intracellular molecules. These include the components of bacterial cell membranes. For instance LPS, a component of the cell wall of gram-negative bacteria, is recognized by TLR-4, while peptidoglycan, lipoproteins and lipopeptide components of gram-positive bacteria are recognized by TLR-2, TLR-1 and TLR-6 respectively [218,219]. In addition, PAMPs could also be extracellular proteins such as flagelin (part of bacterial flagella) which is recognized by TLR-3 [220,221,222]. PAMPs can also be nucleic acids such as bacterial and viral DNA, which consist of unmethylated CPG motifs, and are recognized by TLR-9 [220,221,222]. Beside DNA, single stranded RNA (TLR-7 and TLR-8 ligands) and double stranded RNA (dsRNA) (TLR-3 ligands) contain PAMPs and are mostly derived from virally infected cells [220,221,222].

TLR ligation triggers several intracellular signaling pathways, in particular MyD88-dependent pathways. MyD88 is an adaptor molecule that connects the intracellular domain of TLRs with IRAK4 [223,224,225]. The binding between MyD88 and IRAK-4, results in IRAK-4 phosphorylation inducing the kinase activity in IRAK-1, and the subsequent phosphorylation of IRAK-1 N-terminus [226]. This process of phosphorylation results in TRAF-6 recruitment and binding [226]. Afterward, the IRAK-1-TRAF-6 complex disconnects from the receptor, and at the cell membrane they interact with TAK1, TAB1 and TAB2/3 [226]. As a result, TAB2/TAB3 and TAK-1 are phosphorylated, and translocate to the cytoplasm all together with TRAF-6 [226]. In the cytoplasm, TAK-1 is phosphorylated and as a result the IKK (IκB kinase) complex becomes

phosphorylated followed by NF-κB translocation [226]. IKK is a complex consisting of three subunits two of which are catalytic (Ikkα and Ikkβ) and one of which is regulatory the Ikkγ (also known as NF-κB essential modulator, NEMO) [226]. Activated IKK phosphorylates IκBs (a NF-κB inhibitor) [226]. NF-κB is consist of P65, P52 and P50 and consistently present in the cytoplasm in an inactive form in a complex with its inhibitor IκB [226]. The phosphorylation of IκB by activated IKK results in the polyubiquitylation and proteasome degradation of IκB [225] and the subsequent release and translocation of NF-κB [226].

The phosphorylation of TAK-1 following TLR ligation also triggers the mitogen-activated protein kinase (MAPK) pathways [226]. Three MAPK pathways have been identified: ERK (Extracellular-signal-regulated kinase) pathway, P38 pathway and Jun N-terminal kinase (JNK) pathway [227]. In general, MAPK pathways can be activated either though TAK-1 or though GTPase-mediated pathways that include Rho, RAC, RAS and CDC42. The activation of these molecules leads to the phosphorylation of Mitogen activated protein kinase kinase kinases (MAPKKKs) molecules, such as RAF, TAK (TGF-β-activated kinase) and MEKK (MAPK/ERK kinase kinase) [228]. The phosphorylation of MAPKKKs leads to the phosphorylation of MAPK Kinases (MAPKK) and the subsequent phosphorylation of MAPK (P38, JNK, and ERK). Once MAPK is activated they can translocate into the nucleus [228]. NF-κB and MAPK translocation leads to the transcription of a variety of cytokines and to an up-regulation in the expression of co-stimulatory molecules.

A) NF-κB function and disease

NF-κB activation and the subsequent translocation induces the expression of pro-inflammatory cytokines, chemokines and an up-regulation of co-stimulatory molecule expression [229]. It has been reported that NF-κB can play a pathogenic role in inflammatory diseases such as arthritis. For example, in rheumatoid arthritis NK-κB is found to be overexpressed in the inflamed synovium [230]. Moreover, by bronchial biopsies, asthma patients showed a correlation between the levels of NF-κB activation and the expression of cytokines and adhesion molecules that are associated with the disease [231]. Also, *Helicobacter Pylori* associated gastritis was characterized by an increase in NF-κB activation in gastric epithelial cells [232].

Collectively, these studies suggest that targeting NF-κB could have a beneficial effect on inflammatory disease. However, several studies have revealed that chronic inflammatory conditions can be initiated by inhibiting NF-κB, in particular at epithelial surfaces, where epithelial cells maintain the immune homeostasis through NF-κB activity [233]. Mice that have deficiencies of NEMA in intestinal epithelial cells (IEC) have severe chronic colitis associated with increased levels of pro-inflammatory mediators, immune cell infiltration, and epithelial ulceration [234]. Also, $NEMA^{IEC\text{-}ko}$ mice showed severe intestinal inflammation, however, the underlying mechanism in these studies still unclear [235]. NF-κB remains an attractive therapeutic target for inflammation, but only if its complex role is fully understood [236].

B) MAPK function and disease

On the other hand, different members of the MAPK family are associated with different immune cell functions. For example, ERK has been associated with cell proliferation, meiosis and stress responses [237]. However, P38 appears to be more involved in inflammation and apoptosis [237]. In RA, all MAPK members where found to be activated in the synovial tissue of RA patients, interestingly each member was activated in different location [238]. For example, activated P38 was found in the synovial microvessels and cells lining the synovial layer, while activated ERK was localized in mononuclear infiltrates and cells within the area around the lining of the synovial microvessels [238], suggesting that MAPK members may contribute differently in the disease pathogenesis [239]. Inhibiting ERK activation was associated with a reduction of inflammation in an ear oedema model and osteoarthritis model [240,241]. P38 inhibition has been found to block TNF-α, IL-1 and IL-8 production by monocytes [242,243]. Therefore, blocking P38 activation was found to be associated with a reduction in the clinical severity of RA. Indeed, P38 inhibition is now at phase II trail for RA [239].

C) The role of P38, ERK and NF-κB in IL-10 production.

Activation of P38 and ERK pathways has been associated with not only the production of pro-inflammatory cytokines, but also with the production of anti-inflammatory cytokines such as IL-10 [244]. In a study by Zhang *et al.* it was reported that P38 and ERK inhibition, by the chemical inhibitors SB203580 and U0126 respectively, suppressed IL-10 production. In contrast blocking NF-κB translocation using SN50 up-regulated IL-10 production in recombinant

hemagglutinin B activated macrophages [244]. These results suggest that P38 and ERK play an important role in up-regulating IL-10 production, while NF-κB may play the opposing role [244]. Supporting this hypothesis Reissinger *et al.* reported that macrophages showed down-regulation in P38 and ERK in response to *Bordetella* type III secretion system (machinery that allow the bacteria to insert their proteins into the cell cytoplasm), and this was associated with a decrease in IL-10 and IL-6 production compared to controls [245]. Driessler *et al*. have documented that pre-treating U937 (a human monocyte line), or PBMCs, with IL-10 in the presence of TNF-α blocked nuclear translocation of P65 and induced nuclear accumulation of P50 [246]. Also, it has been reported that NF-κB dependent luciferase activated LPS RAW 264.7 (murine macrophage-like cell line) was significantly inhibited in LPS-activated RAW 264.7 in the presence of IL-10 [247]. P38 has also been reported to up-regulate NF-κB, in a study by Saccani *et al.* P38 was found to play a crucial role in inducing inflammation and regulating NF-κB recruitment in LPS stimulated DCs [247]. Collectively, these studies reveal that ERK activation is associated with IL-10 production, while NF-κB inhibition up-regulates IL-10 production. P38 involvement in IL-10 expression is controversial as some studies suggest that P38 activation is associated with IL-10 down-regulation, whereas other studies found the opposite.

D) The role of P38, ERK and NF-κB in CD80 and CD86 expression.

With regard to CD80 and CD86 expression, it has been reported that blocking P38 and NF-κB with SB203580 and BAY-117082 respectively, inhibited CD80 and CD86 up regulation in OK-432 (a Streptococcal preparation) activated DCs, while blocking ERK with PD98059 was associated with CD80 and CD86 up regulation [249]. In agreement with this study, a study by Arrighi *et al.* showed that blocking P38 inhibited the high expression of CD80, CD86, CD40 and HLA-DR that is induced in LPS and TNF-α activated immature DCs, while blocking ERK did not affect the expression of any of these molecules [250].

1.5.2 TLRs and B cells

One of the unique characteristics of B cells is the dual expression of antigen-specific B cells receptors (BCR) and TLRs that permit B cells to integrate danger and Ag-specific signals [251]. TLR signals in B cells can play an important role in modifying their response in terms of cytokine secretion, antibody production and antigen presentation [251]. B cells can respond in a T cell dependent or independent manner, FO B cells represents the arm of the T-dependent immune responses, while B-1 and MZ B cells are the main players in T cells independent responses [251]. Therefore, MZ and B-1 B cells can be viewed as innate like cells that rapidly produce antibodies in response to pathogens without T cell help [251].

TLRs ligation induces rapid proliferation and antibody production, in particular the production of IgA (B-1 cells) and IgM (MZ B cells) [252,253,254], in a T-independent fashion. MZ and B-1 B cells have a functionally stronger response

than FO B cells to TLR engagement; MZ and B-1 B cells express higher levels of co-stimulatory molecules and produce higher levels of IL-10 than FO B cells in response to TLR ligation [253,254,255,256]. Moreover, following TLR ligation MZ B cells are more efficient antigen presenting cells than FO B cells [253,254,255,256]. In addition, TLRs can regulate MZ and B-1 B cell localization and migration [257]. MZ B cells, for instance, express sphingosine-1 phosphate receptor 1 (S1PR1) that retains the MZ B cells in the MZ [257]. Administration of a TLR-4 ligand (LPS) *in vivo* leads to MZ B cells migration into the follicles through S1PR1 down regulation [258]. Similarly B-1 B cells, which express high levels of integrins, down regulate their integrin and CD9 expression following TLR ligation leading to their migration from the peritoneal cavity to site the of invasion to initiate fast and local antibody responses [259].

Although TLR ligation is of greater importance to B cells involved in T-independent responses, T dependent B cells responses can also be influenced by TLR ligation [252,253,260,261]. Activated FO B cells form germinal centers where they differentiate into either plasma or memory B cells [251]. GC B cells have been found to be more responsive to TLR ligation and express higher levels of MyD88 mRNA than FO B cells, suggesting that GC B cells are more sensitive to TLRs engagement [262]. Also, TLRs have been suggested to play a role in plasma cell differentiation [251].

TLR mediated cytokine secretion by B cells can alter $CD4^+$ T cell polarization. Recently, Miles *et al.* found that apoptotic cells (AC), which express chromatin complexes and stimulate TLR-9, when adoptively transferred protected the mice from arthritis, unlike the transfer of ACs treated with DNase, which

removes TLR-9 ligand, that did not confer protection [264]. In addition TLR-$9^{-/-}$ mice were not protected from EAE upon AC-administration, suggesting a role for TLR-9 in mediating protection from disease [264]. Moreover, protection from EAE by AC treatment was restored by the adoptive transfer of WT but not TLR-$9^{-/-}$ B cells together with AC, suggesting that TLR-9 is essential for B cells to maintain self-tolerance in heath [264].

1.5.3 The interaction of Gal-1 with intracellular signalling molecules

The role of Gal-1 in TLR function or signaling has not received much attention. A study by Fuerter *et al.* investigated the intracellular pathways that lead to Gal-1 expression following T cell activation [265]. T cell activation via TCR/CD3 signals involves Lck and Fyn tyrosine kinase to initiate the pathway; blocking them with PP1 (Src family kinase inhibitor) inhibited Gal-1 expression in activated T cells, suggesting that Lck and Fyn activation are important for Gal-1 activation and expression [265]. This study also investigated the down stream events of Lck and Fyn, which include ERK and P38 phosphorylation, and found that by blocking them lymphoproliferative responses and Gal-1 expression were inhibited [265]. These results suggest that Gal-1 expression by activated T cells requires ERK and P38 [265].

It has recently been reported that blocking NF-κB, but not P38 or ERK, down-regulated Gal-1 expression particularly in Th1 cells [266]. This study also reported that exogenous Gal-1 has an inhibitory effect on NF-κB activation [266]. Thus Gal-1 expression is regulated by NF-κB activity, and the expression of Gal-1 can lead to the inhibition of NF-κB [266]. It has been documented that

Gal-1 can interact with Ras at the cell membrane and activate the ERK pathway [187]. These studies indicate that Gal-1 interacts with a number of molecules that are involved in TLRs signaling pathways. These studies have so far all been carried out in T cells and the role of Gal-1 in B cell signaling pathways is as yet not well defined.

1.5.4 TLRs and transplantation

As mentioned earlier, transplant surgical process is associated with ischemia-reperfusion injury that leads to the release of DAMPs [267,268]. The release of DAMPs activates and recruits the innate immune cells through their TLRs [267,269]. Briefly, released DAMPs activate donor or recipients APCs via their TLRs [268]. Activated APCs amplify the allo-responses through the production of cytokines, chemokines and growth factors that recruit neutrophils and activate monocytes to infiltrate the graft [268]. In addition, APCs up-regulate the expression of their surface co-stimulatory molecules and activate the adaptive immune cells [268]. All together they induce both tissue injury and the release of more DAMPs that might promote chronic rejection via the activation of cells expressing TLRs [268].

1.6 The Hygiene Hypothesis

The hygiene hypothesis is the theory that there is a link between the increase in the rate of allergies and autoimmune diseases and the decrease in exposure to infectious agents, parasites and symbiotic microorganisms, such as an alteration in gut microbiota during child hood, which in turn leads to an altered development of the immune system [219]. This hypothesis has re-shaped our understanding of the role of microbes in normal immune function, suggesting that microbes can be beneficial for health and immune function [220]. Several studies have reported that commensal microbiota, in particular some gut microbiota, may influence the development of immune responses and protect from diseases [221,222]. A study by Shimomura *et al.* reported the influence of housing mice in CV and SPF facilities on the regulatory function exerted by B-1 B cells in chronic colitis [274]. They found that maintaining TCR-$\alpha^{-/-}$ mice, in which chronic colitis is mediated by IL-4 producing Th2 T cells, in CV facilities suppressed colitis, and that B cells in TCR-$\alpha^{-/-}$ mice were more activated under CV environment compared to B cells obtained from mice in SPF facilities [274]. Moreover, they found that the adoptive transfer of B-1 B cells isolated from mice kept in the SPF facility into double knocked out mice (B cells$^{-/-}$ and TCR-$\alpha^{-/-}$) housed in the CV facility significantly suppressed the disease compared to the control, which received PBS [274]. These data suggest that the protection observed from Th2-mediated colitis by housing the mice under non-hygienic conditions is mainly due to the regulatory role that B-1 B cells play under the response to microbial flora [274]. Recently, in an allergic model, it has been demonstrated that treating mice with antibiotics affected their immune make up,

with higher concentrations in serum IgE and circulating basophil (mediators of allergy) [220]. Moreover, allergic responses were more severe in mice treated with antibiotics and exposed to dust allergen [220]. In this study, they also investigated the link between high levels of circulating basophils and serum IgE by testing the level of circulating basophil in RAG1 (B cell deficient) mice and mice where IgE was neutralized; they found no increase in the level of basophils suggesting a link between B cells [220]. Also, they have found that the commensal microbiota influence on B cells to produce IgE is MyD88 dependent, where $MyD88^{-/-}$ mice (without antibiotic treatment) showed the same high levels in IgE and basophil observed in germ free and antibiotics treated mice, which also was seen in B cells $MyD88^{-/-}$ mice [220]. However, how and where B cells come in contact with microbial product is yet to be determined [220].

Chapter 2- Materials & Methods

2.1 Materials

2.1.1 Antibodies & Reagents

Table 1

Extracellular surface Antigens Anti-mouse Antibodies				
Antibody To	**Conjugate**	**Clone**	**Isotype**	**Manufacturer & Concentration (ratio/50μl)**
CD19	FITC	1D3	Rat IgG2a,κ	eBiosciences 1:100
	APC			
	Pacific Blue (PB)			
CD21	FITC	4E3	Rat IgG2a,κ	eBiosciences 1:100
	PB	7E9		Biolegend 1:100
CD23	PE-cy7	B3B4	Rat IgG2a,κ	eBiosciences 1:200
CD24	PE	M1/69	Rat IgG2b,κ	eBiosciences 1:800
CD38	PE	90	Rat IgG2a,κ	eBiosciences 1:200
CD95	APC	15A7	Ms IgG1,κ	eBiosciences 1:100
GL7	FITC	GL7	Rat IgM	eBiosciences 1:100
CD4	FITC	GK1.5	Rat IgG2b,κ	eBiosciences 1:100 1:200 1:100
	PE-cy7			
	PB			

Extracellular surface Antigens Anti-mouse Antibodies				
Antibody To	**Conjugate**	**Clone**	**Isotype**	**Manufacturer & Concentration (ratio/50μl)**
CD4	PE	RM4-5	Rat IgG2a,κ	eBiosciences 1:200
CD8	APC	53-6.7	Rat IgG2a,κ	eBiosciences 1:100
CD25	FITC	7D4	Rat IgM,κ	BD Biosciences 1:100
TCR-β	PE	H57-597	Ar Ham IgG	eBiosciences 1:200
mCD1d	APC	Loaded tetramer	---	Gift from NIH 1:100
NK1.1	FITC	PK136	Ms IgG2a, κ	eBiosciences 1:100
Tim-1	PE	RM11-4	Rat IgG2b,κ	eBiosciences 1:200
IgG	FITC	Poly-clonal	-----	Sigma 1:100
CD40	PE	IC10	Ar Ham IgG2,κ	eBiosciences 1:200
CD40L	PE	MR1	Ar Ham IgG3,κ	BD Biosciences 1:200
I-A^b	PE	AF6-120.1	Ms IgG2a, κ	eBiosciences 1:200

Table 1 continue

Extracellular surface Antigens Anti-mouse Antibodies				
Antibody To	**Conjugate**	**Clone**	**Isotype**	**Manufacturer & Concentration (ratio/50μl)**
K^d	PE	SF1-1.1	Ms IgG2a, κ	eBiosciences 1:200
CD80	PE	16-10A1	Ar Ham IgG2,κ	BD Biosciences 1:200
CD86	FITC	GL1	Rat IgG2a,κ	BD Biosciences 1:100
CD69	APC	H1.2F3	Ar Ham IgG	eBiosciences 1:100
CD62L	FITC	MEL-14	Rat IgG2a,κ	eBiosciences 1:100
TLR-4	PE	UT41	Ms IgG1	eBiosciences 1:200

Table 1 continue

Table 2

Intracellular Antigens Anti-mouse Antibodies				
Antibody To	**Conjugate**	**Clone**	**Isotype**	**Manufacturer & Concentration (ratio/50μl)**
IL-10	APC	JES5-16E3	Rat IgG2b,κ	eBiosciences 1:100
TNF-α	APC	MP6-X122	Rat IgG1,κ	eBiosciences 1:100
IFN-γ	APC	XMG1.2	Rat IgG1,κ	eBiosciences 1:100
P38	2^0 is required	3D7	Rabbit IgG	Cell signaling 1:25
ERK1/2	2^0 is required	T202/T204	Rabbit IgG	Cell signaling 1:200
NF-κB	2^0 is required	C-20	Rabbit IgG	Santa Cruz Biotechnology 1:20
TLR-9	FITC	M9.D6	Rat IgG2a,κ	eBiosciences 1:100

Table 3

Cell culture antibodies				
Antibody To	**Conjugate**	**Clone**	**Isotype**	**Manufacturer & Concentration (μg/ml)**
CD86	Purified	P03	Rat IgG2b,κ	BD Biosciences 5 μg/ml
CD80	Purified	16-10A1	Rat IgG1,κ	BD Biosciences 5 μg/ml
IL-10	Purified	JES5-2A5	Rat IgG1,κ	BD Biosciences 10 μg/ml
IL-10 Receptor	Purified	1B1.3a	Rat IgG1,κ	BD Biosciences 10 μg/ml

Table 4

Fluorescent in-situ hybridization (FISH-Flow) Probs			
Probe	**Targeted Position**	**Sequence frm 5' to 3' end**	**OPD code**
NON 338		ACATCCTACGGGAGGC	NA
Erec 482	482-500	GCTTCTTAGTCARGTACCG	S-*-Erec-0482-a-A-19
Bac 303	303-320	CCAATGTGGGGGACCTT	S-*-Bac-0303-a-A-17
Bif 164	164-181	CATCCGGCATTACCACCC	S-G-Bif-0164-a-A-18
Lab 158	158-178	GGTATTAGCAYCTGTTTCCA	S-G-Lab-0158-a-A-20

2.1.2 Anti-CD40 (FGK45 Hyridoma) culture and purification

$1x10^7$ cells/ml of FGK45 hybridomas were kept in heat inactivated FCS (Foetal Calf Serum, Sera Laboratories International Ltd) and 10% DMSO (Dimethylsulfoxide, Sigma-Aldrich). After defrosting the cells (37^{o}C water bath) for culture, cells were washed twice with culture media. Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D6429) containing 5%FCS, 2mM L-glutamine, 100U/ml penicillin and 100U/ml streptomycin and sterile cell culture flasks were then used to culture hybridomas. Once the hybridomas have expanded and adhered to the flask wall, the flask was then filled with (~500ml) of culture media, and hybridomas were left to produce antibodies until the media was consumed (flask upright). After collecting the supernatants, the supernatants were centrifuged, passed via sterile filter (0.22μM mess), and stored at 40^{o}C.

Culture supernatants were run over a Prosep (Protein-G column (Bioprocessing, UK) for monoclonal antibodies purification. The column was prepared by running ~10 column volumes of PBS (pH 7.4) containing 0.5M Glycine. Then the supernatants were passed through the column followed by running 10 column volumes of PBS containing 0.5M Glycine. 0.1M glycine/HCL pH2.2-3.5 solution were then used to elute the protein and to be collected as a fractions of 5ml and tested for protein content by spectrophotometry. After pulling and dialyzing the protein containing fractions against PBS pH 7.4 for 24hrs, the antibody solution was filtered and the concentration was measured by spectrophotometry. The antibodies were then tested by its ability to induce B cell cytokines production.

2.1.3 Mice and isolation of mouse cells

2.1.3.1 C57BL/6 and Gal-1$^{-/-}$ mice

Wild type male and female C57BL/6 (H-2K^{b}) mice and BALB/C (H-2K^{d}) female mice were purchased from Harlan Laboratory, and the donor B6 K^{d} (H-2K^{b}) mice were a generous gift from Dr. R Pat Bucy (University of Alabama at Birmingham, Birmingham, AL, USA). F.Poirier provided Gal-1$^{-/-}$ mice. All mice were kept either in a Specific Pathogens Free (SPF) facility, or in a Conventional (CV) facility. μMt mice were provided by Jean Langhovne (NIMR, Mill Hill, London) and kept in SPF facility.

The Home Office (UK) approved all mouse protocols utilized in this study.

2.1.3.2 Genotyping Gal-1$^{-/-}$ mice

DNA purification from mice tails

Samples were obtained from the tips of Gal-1$^{-/-}$ (Gal-1$^{-/-}$) mice tails. Tails were then placed in sterile 1.5ml microfuge tubes with 300μl DirectPCR Lysis Reagent (Viagen Biotech, Inc.) and 0.2mg/ml proteinase K (sigma, UK). After overnight incubation at 55^{o}C, the lysates were incubated for 45mins at 85^{o}C. Next, the samples were centrifuged at 1400rpm and 1μl was used for genotyping (stored at -20^{o}C).

Primers

3?NeoGal1 5'GTGGTCTTGACAAAAAGAACC	
5?Gal1 5'CTCAGTGGCTAGATCTGTAAAATGG	55.3^{o}C
3?Gal1 5'TTCTTTGACATTTGAACCCTATACC	

PCR Programme

Step 1- 94°C 2mins

Step 2- 94°C 30s

Step 3- 55°C 30s | 30 cycles

Step 4- 74°C 1min

Step 5- 72°C 5mins

Step 6- 4°C pause

PCR Protocol

PCR strips (Abgene) were used to carry out the PCR reactions. The PCR mix (50μl) contained 1μl tail lysate, 1.5mM MgCl2 (Promega), 1x Thermophilic Polymerase DNA Buffer (Promega), 200μM dNTPs (Promega), 0.4μM primers (Sigma) and 2.5 units of GoTaq (DNA polymerase (Promega). 1:5 diluted PCR products with DNA loading dye and run on 1% agarose gel (1x Tris-acetate-EDTA (TAE) buffer (Promega)) containing 3μl ethidium bromide/100ml.

2.1.4 Lymphocytes and splenocytes isolation from mice spleens and lymph nodes

Spleens and lymph nodes (LNs) were mashed through 70μm nylon cell strainer (BD Falcon), and the splenocytes were haemolysed with ammonium chloride potassium carbonate (ACK) buffer. Subsequently, the cells were washed extensively and re-suspended either in FACS buffer (1x Phosphate Buffered Saline (PBS) with 0.4% fetal calf serum (FCS) & 0.4% ethylenediaminetetraacetic acid (EDTA)), or complete media, and either stained with the mAbs listed above or cultured. The entire process was carried out under sterile conditions.

2.2 Methods

2.2.1 Cell culture

Complete RPMI 1640 (Sigma-Aldrich, UK) were used for all cell culture experiments, supplemented with 10% FCS and 100U/ml penicillin/streptomycin (Life Technologies) at 37°C, 5% CO_2.

2.2.1.1 Culture for intracellular staining and cytokines detection

Cells were cultured at concentration of 2-5x10^5 cells per well for 48hrs. PMA (50ng/ml), Ionomycin (250ng/ml) and brefeldin A (1:1000) were added for the last 4 hours of culture. All cell cultures were carried out in U-bottom 96 plates at final volume of 200μl/well.

2.2.2 Magnetic sorting for cell subsets isolation

Untouched mouse splenic B and $CD4^+$ T cells were isolated using magnetic labeled beads. Cells were purified from freshly isolated splenocytes. All reagents were purchased from Milteny.

2.2.2.1 CD43 splenic B cells negative selection (Miltenyi kit: 130-049-801)

Splenocytes were washed in 50ml falcon tubes with MACS buffer (1xPBS containing 0.5% FCS, 2mM EDTA, kept on ice), centrifuged (500g, 4°C, 5mins), and the supernatant carefully decanted. Every 10^7 cell were then re-suspended in 90μl of MACS buffer plus 10μl of CD43 beads, mixed, and incubated for 15mins at 4^0C. Subsequently, cells were washed with MACS buffer 1ml/10^7 cells, centrifuged, and supernatants were decanted. Next, cells were re-

suspended in 500μl MACS buffer per 10^8 cells, and passed through MACS LD column placed in QuadroMACSTM magnetic fields. LD columns were rinsed with 2ml MACS buffer, then cell suspension were applied through the column, and the column were washed 3 times with 2ml MACS buffer. Cells were then collected in 50ml falcon tube and counted.

2.2.2.2 CD4 splenic T cells negative selection (Invitrogen: 114.16D)

Splenocytes were washed in 50ml falcon tubes with MACS buffer, centrifuged, and the supernatant carefully decanted. Every 10^7 cell were then re-suspended in 100μl of MACS buffer plus 20μl of FCS and 20μl antibody mix, mixed, and incubated for 20mins at 4^0C. Subsequently, cells were washed with MACS buffer 2ml/10^7 cells, centrifuged, and supernatant were decanted. Next, cells were re-suspended in 800μl MACS buffer per 10^8 cells, and 200μl of depletion Dynabeads and incubated for 15mins at 4^0C on a rotator. The depletion Dynabeads (200μl/10^7 cells) were washed by adding the same volume (200μl/10^7 cells) of MACS buffer, mixed and placed in magnet for 1min, and the supernatant were discarded, beads were then re-suspended in the same initial volume of MACS buffer. Subsequently, cell suspension were mixed and 1ml MACS buffer per 10^7 cells were added. Then the tube was placed in magnet (Dynal-MPC-1) for 2mins, and supernatants were removed (this step was repeated twice). Cells were then collected in 50ml falcon tube and counted.

The depletion Dynabeads (200μl/10^7 cells) were washed by adding the same volume (200μl/10^7 cells) of MACS buffer, mixed and placed in magnet for 1min,

and the supernatants were discarded, beads were then re-suspended in the same initial volume of MACS buffer.

2.2.3 Isolation of B cell subsets by FACS sorting

Purified splenic B cells were suspended in 1ml MACS buffer per $50x10^6$ cells (50ml falcon tube). Subsequently, cells were stained with anti-CD21-FITC (1:100), anti-CD24-PE (1:1600) and anti-CD23-PE-cy7 (1:200). Cells were then incubated for 30mins at 4^0C, and washed 2 times with MACS buffer. Next, every $50x10^6$ cells were re-suspended in 1ml of FACS buffer. DAPI (1:100) was added to the cells just before starting the sort to exclude dead cells.

Sorting took place on a BD FACSAria (BD Biosciences). Doublets were gated out. Sorted cells were collected into polypropylene tubes containing sterile 30% FCS/PBS. Examples of sorting gating strategies and post-sort B cell subsets are given in figures 3.1.

2.2.4 *In vitro* experiments

2.2.4.1 Allo-DCs generation

BALB/c mice, which express H2-k^d, ethanol sterilized femurs and tibias Bone Marrow (BM) were flushed to obtained fresh BM cells. The cells were subsequently passed through 70μm cell strainer (BD Falcon) to obtain single cell suspension. Next, cells were incubated with ACK (ammonium chloride potassium carbonate) lysing buffer for 10mins to haemolysed erythrocytes. BM cells were incubated for 30mins with the supernatants isolated from 53-6.7, YTS 191, M5/114, RA3-3A1 rat anti-mouse hybridoma cultures. Subsequently, the cells were washed twice with RPMI and incubated for 30mins at 4^oC on roller

with sheep anti-rat Dynabeads and separated through magnetic field. The cells were then washed and plated in 24 well plate at $1x10^6$ cells/well in 1ml complete media supplemented with 20ng/ml murine recombinant granuloctes/macrophage colony-stimulating factor (GM-CFS) (R&D, UK). Plates were swirled gently and the fresh complete media containing GM-CFS were added on day 2 and 4 of culture. One day before using the allo-DCs, LPS (E.coli, Enzo Life Sciences, UK) were added to induce maturation. After 7 days of culture, allo-DCs were collected, washed three times, and irradiated for 10mins.

2.2.4.2 *In vitro* B cell subsets suppression assays

Isolated $CD4^+$ T cells were activated through plate bound anti-CD3 antibodies (eBiosciences), CD3/CD28 activator beads (Invitrogen, UK), or irradiated allo-DCs. Anti-CD3 antibodies were diluted (1.5μg/ml) in PBS, and incubated for 2 hours in round bottomed 96 well plates at 37℃. The plate was then washed with PBS. T cells were re-suspended in RPMI ($1.5\text{-}2x10^5$ cells/well) and added to the coated plate, or to non-coated plate in the presences of allo-DCs or CD3/CD28 activator beads with non-activated or activated sorted B cell subsets ($1.5\text{-}2x10^5$ cells /well) with or without anti-CD80 Abs (5μg/ml), anti-CD86 Abs (5μg/ml), anti-IL-10 Abs (10μg/ml), anti-IL-10 receptor (10μg/ml), lactose (10mM/ml) (sigma,UK), or sucrose (10mM/ml) (sigma,UK) for 48 hours.

For the activated sorted B cell subsets, B cell subsets were pre-activated with CpG/LPS (1μg/ml and 50nM/ml, respectively) for 4 hours in round bottomed 96 well plates at 37℃, washed 3 times with PBS, and added together with T cells into anti-CD3 antibodies.

2.2.4.3 B cells activation system

Magnetically isolated B cells ($2x10^5$ cells/well) were re-suspended in RPMI 1640 (10% FCS), and activated for 48 hours with anti-CD40 antibodies (10μg/ml), anti-CD40 antibodies plus LPS (1μg/ml) from E.coli (Enzo Life Sciences), anti-CD40 antibodies plus CpG-B (1nM/ml) (ODN 1826, ALEXIS Biochemicals) and CpG plus LPS. After 48 hours of incubation, the cells were labeled with anti-CD19-APC, anti-CD80-PE and anti-CD86-FITC antibodies and stained for intracellular cytokines.

2.2.5 Mouse experiments

2.2.5.1 Skin transplant

B6 mice (expressing MHC I H-$2K^b$) received on their back transplants of 1cmx1cm bit of tail skin from either MHC I mismatched transgenic C57BL/6 mice that express H2-k^d (B6-Kd) or B6 mice. CD8 cells were depleted by IP injection of anti-CD8 antibody (150μg) on day -1, 0 (day of skin graft), +1 and every 7 days following the skin graft. Rejection was evaluated 7 days post transplant on the basis of escharing (Figure 3.11).

2.2.5.2 Induction of tolerance to allograft

B6 mice seven days before receiving a skin graft from B6 K^d, receive $25x10^6$ donor splenocytes (DST) + 250 μg MR1 (anti-CD40L antibody) plus an additional injections of 100μg MR1 three days before the graft, on the day of graft and four days subsequently.

2.2.6 Flow cytometry

Tissues were mashed through 70µm nylon cell strainer, and the erythrocytes were haemolysed with ACK buffer. Subsequently, the cells were washed extensively and re-suspended ($2x10^6$ cells/well) in FACS buffer, and stained with the mAbs at a final volume of 50µl, and washed in 200µl FACS buffer. The cells were acquired using BD LSR II, and sorted by BD FACSAria II cytometer. Analysis was performed by Flowjo software. The staining was performed in 96 well U-bottomed plates. All *in vitro* experiments gating were based on live cells using fluorescence-based LIVE/DEAD assay (Invitrogen, UK).

2.2.6.1 Surface Staining

In vitro cultured and stimulated cells, or freshly isolated splenocytes were washed twice with PBS at 500x g for 5mins. Then, to check the viability of the cells live/Dead Fixable blue Dead cell staining kit (invitrogen) were reconstituted according to the manufacturers protocol, and 200µl were added per well (for every 5 wells, 0.5µl of the reconstituted dye was diluted in 1ml PBS) and incubated for 30 minutes at room temperature in dark. Subsequently, cells were washed three times with PBS and supernatants were discarded and cells were stained with relevant conjugated anti-mouse antibodies. After 20mins of incubation at 4^oC, cells were washed twice, and fixed with 1x CellFix (1:10 diluted in dH_2O) (BD, UK) for 10 to 15mins at 4^oC. Cells were then washed twice and re-suspended in 200µl FACS buffer and acquired using BDLSR II or BDFortessa, or carried through Fix/Perm steps for intracellular staining (see below).

2.2.6.2 Intracellular Staining for the detection of cytokines

After initial cells surface staining as described above, cells were fixed via 20mins incubation at 4^{o}C with 50μl of Cytofix (eBiosciences). Cells were then washed with 1x Permwash (10x Permwash in dH20) (eBiosciences), and incubated subsequently with 200μl of 1x Permwash for another 20 minutes. Next, Supernatant were discarded after spinning the cells at 500x g for 5mins, and re-suspended 50μl of relevant anti-mouse Abs diluted in 1x Permwash. Following 20mins of incubation at 4^{o}C, cells were washed twice with 1x Permwash and once with FACS buffer. BD LSR II of BDFortessa was used to acquire the cells; analysis was done by Flowjo software.

2.2.6.3 Antibodies detection

Total IgG was measured in the sera of B6 mice that had received B6-K^{d} skin grafts by incubating the sera with H2-K^{d} expressing BALB/c splenocytes. Binding of anti-K^{d} antibody to H2-K^{d} on CD3^{+} T cells was then detected by flow cytometry. The BALB/c splenocytes (2.5x105 cells/well) were blocked by anti-CD16/CD32 antibodies (eBiosciences) and then labeled by anti-CD3-PE followed by adding diluted sera (1:10; 1% Bovin Serum Albomin (BSA)/ PBS). After 20 minutes of incubation, total anti-IgG-FITC antibodies (Sigma Immunochemicls) were incubated with the cells for 20 minutes. The cells were acquired by BD LSR II or BDFortessa and analyzed by Flowjo software.

2.2.6.4 fluorescent in-situ hybridization (FISH-Flow)

Stool samples were collected from mice either by stressing the animals or directly after culling them. Samples were stored at -80^{o}C. Samples were first fixed by adding 500μl PBS to 0.05g samples, and 3-4 glass beads homogenise with tissue lyser for 5mins. The sample was then aliquot (200μl) and 600μl of 4% paraformaldehyde solution (PFA) was added, and incubated overnight at 4^{o}C. Subsequently, the samples were permeabilised by spinning the samples at 500x g for 1min to remove debris, 250μl of the fixed bacterial solution were then added to 200μl PBS, centrifuge at 8000x g for 3mins and the supernatants were discarded. Next, 1ml of Tris-EDTA were added followed by 3mins centrifugation at 8000x g. After discarding the supernatants, 1ml of Tris-EDTA with lysozyme (20mg lysozyme in 20ml Tris-EDTA) were added, mixed, incubated for 10mins at room temperature and centrifuge at 8000x g for 3mins. The supernatants were discarded, 1ml of PBS were then added, samples vortexed and centrifuged as previous. After permeabilisation the samples were hybridised. First, 1ml of hybridisation buffer (NaCl 5M, Tris HCl 1M pH8, SDS 20%, Formamide 12ml and 30ml dH_2O) were added, vortexed, centrifuged as above and the supernatants were discarded. 240μl hybridisatiion buffer were then added and 40μl of samples were transferred into wells. 10μl of the relevant probe (section 2.2) were added and incubated overnight at 37^{o}C. Next morning, 150μl of hybridisation buffer were added, centrifuged at 4000rpm for 10mins, and the supernatants were discarded. Subsequently, the samples were washed by 200μl washing solution (NaCl 5M, Tris HCl 1M pH8, EDTA 0.5M pH8, SDS 20% and 47.8ml dH_2O), mix, and incubate for 20mins at 39^{o}C. The plate was

then centrifuged as above and 200μl PBS was added and the samples were acquired using BDLSR II.

2.2.7 ELISA

2.2.7.1 IL-10 ELISA

After cells culture and stimulation, supernatants were collected before adding PMA, ionomycin and brefeldin A, and kept at -80^{o}C until use. 100μl/well of IL-10 capture antibody was incubated overnight at 4^{o}C at concentration of 5μg/ml in PBS/Tween (PBS/ 0.05% Tween-20) in 96 well MaxiSorp plates (NUNC) to coat the plate. Subsequently, plates were washed 5 times with PBS/Tween and blocked with 200μl per well 2% BSA in PBS for 1 hours at room temperature (RT). After washing the plates 5 times with PBS/Tween, recombinant IL-10 standards were serially diluted (1:2) with a top concentration of 4000pg/ml and bottom standard of 31.25pg/ml, and 100μl of 1:4 diluted supernatants and prepared standards were added and incubated for 2 hours at RT. Plates were then washed 5 times with PBS/Tween and 100μl of biotinylated IL-10 detection antibody (0.5-2μg/ml) were added and the plates were incubated 1 hours at RT. After 5 washes, 100μl/well of streptavidin-horseradish peroxidase was added and incubated for 30mins at RT. Plates were washed for 14 times, and 100μl of substrate solution (3,3',5,5'-tetramethylbenzidine (TMB)) were added for 11mins at RT, and the reaction were stopped by adding 50μl of stop solution (450ml dH_2O plus 20ml H_2SO_4), and the plates were read at 450nm.

2.2.7.2 Flowcytomix

Standards and reagent were made up according to manufacturers protocol (eBiosciences, UK). Supernatants were collected after stimulating cells for 42hours with LPS, CPG and anti-CD40 mAb (section 2.2.4.3), and stored at -80^{o}C until use. 25μl of Standard mixture dilutions 1 to 7 and samples were transferred into tubes plus an additional blank tube (25μl assay buffer), and 25μl of beads mixture and 50μl of Biotin-conjugate mixture were added to all tubes, mixed, and incubated for 2 hours at RT. Subsequently, 1ml of assay buffer were added and the tubes were centrifuged for 5mins at 200xg (this step was repeated). After discarding the supernatants, 50μl of streptavidin-PE solution were added, mixed and incubated at RT for 1 hour in dark. Tubes were washed twice as previously, and 500μl assay buffer were added and the samples were acquired using BDLSR II. Flowcytomix pro 2.4 software was used for analysis. The kit was a grateful gift from eBiosciences.

2.2.8 Western Blot

Isolated B cells were washed with PBS, and the pellets were stored at -80°C. The dry pellets were lysed in 50μl lysis buffer per 5x10^{5} cells for one hour on a rotator at 4℃. The lysis buffer consist of 20mM Tris-base (sigma), 5mM EDTA (Gibco BRl), 1% NPHO, 2.5% protease inhibitor cocktail (sigma), 0.5% PMSF (sigma) and 0.05% soyabean (sigma). After one hour of incubation, the lysed cells were spun down in a centrifuged (13000rpm/5 minutes), and the supernatant was transferred into a new tube. The lysates were then run overnight through a sodium dodeyl sulfate-polyacrylamide gel by

electrophoresis (SDS-PAGE). The proteins were then transferred into immobilon-P transfer membrane (Millipore), using a semidry transfer system. The membranes were subsequently probed with mouse anti-human galactin-1 mAb (Biotech) in PBS with 5% milk + 0.2% Tween 20 (sigma) for 1hour. Goat HRP-conjugated rabbit anti-mouse IgG (Bio-RAD) were then added and incubated for 1hour, and developed with ECL plus western blotting detection reagent (GE Healthcare). Regulatory T cells were obtained from cell line, and used as a positive control.

2.2.9 Signaling pathways protocols

2.2.9.1 Cells activation and fixation

Isolated B cells were washed with cold PBS, and $0.5x10^6$ cells (50μl) per well were stimulated either with 100μl LPS (2μg/ml) or CPG (300nM/ml) at 37°C either for 0, 10,30, 45, and 60mins (for P38 & ERK pathways) or 0, 20, 45 and 90mins (NF-κB pathway). The cells were then fixed with 50μl of 4% formaldehyde (10% stock, sigma, UK) on ice for 10mins, centrifuged and washed. Cells were then suspended in 1:100 diluted anti-CD19 mAbs for 30mins.

2.2.9.2 P38 & ERK

After staining with anti-CD19 mAb, the cells were permeabilised in 200μl of 90% methanol (sigma, UK) for 30mins on ice, washed twice with incubation buffer (1% BSA in PBS), and the primary antibodies (p38 and ERK) were then added for one hour at RT. After washing twice, the secondary antibodies were added for 30mins. Cells were then washed twice and acquired by BDLSR II.

2.2.9.3 NF-κB

After staining with anti-CD19 mAb, the cells were incubated for 20mins with anti-NF-κB Ab diluted in prepared permeabilised buffer (0.1% triton X-100, 2% FCS, 0.1% azide and PBS). Then, the cells were washed and re-suspended in 50μl of the secondary antibodies for 15mins at room temperature. Finally, cells were washed and 50μl PBS and DAPI (1:100) were then added just before acquiring through Image stream. The results were analyzed using IDEAS software.

Chapter 3- Results 1

Chapter 3

B cells are mainly known for their ability to produce antibodies, but they also contribute to antibody-independent mechanisms such as antigen presentation and cytokine production [125]. Therefore, they have the potential to shape the immune response [275,276,277]. B cells are in fact involved in the early stages of Th subsets differentiation [260]. A subpopulation of B cell, T2-MZP B cells, has been found to play a protective role in various murine models of chronic inflammation [146,149]. In arthritis, mice with active form of the disease (DBA/1 mice) were protected upon the adoptive transfer of T2-MZP B cells isolated from naïve mice [146]. In lupus, the adoptive transfer of anti-CD40 mAb activated T2-MZP B cells *in vitro* improved MRL/ipr mice survival rate [149]. These studies have suggested that T2-MZP B cells mainly act through the production of IL-10 [146,149].

Gal-1 is classified as immunoregulatory molecules that can regulate cell growth, proliferation, mitosis and apoptosis as well as many other functions [187,190,191]. For example, it has previously been shown that Gal-1 expression is up regulated following Treg cell activation through their TCR receptor and that Gal-1 plays a key role in Treg function [196]. Moreover, it was found that blocking Gal-1 by anti-Gal-1 mAbs *in vitro* inhibited the suppressive function of the Treg cells in humans [196]. Additionally, other studies have also shown that Gal-1 can play a key role in B cell function, in term of their immunoglobulin production [205], development [206], and their influence on T cells [207]. For example, in Trypoansoma cruzi infection model, IFN-γ, but not IL-10 production by Con-A activated T cells was significantly decreased upon

adding Gal-1 purified from infected B cells [207].

The aim of this chapter was to examine whether T2-MZP B cells had a role in transplantation, and explore the possible mechanisms underlying this potential including Gal-1 involvement.

Note

1- For all the results in chapter 3, 4 and 5 the cells counts per FACS acquisition was between $20x10^3$ to $50x10^3$ cells.

2- Emilie Stolarczyk did the FISH experiment in this chapter.

3.1 B cells isolated from SPF mice were unable to suppress $CD4^+$ T cell TNF-α expression *in vitro*.

T2-MZP B cells with a regulatory function and which are able to suppress $CD4^+$ T cell cytokine production, in particular the expression of TNF-α, have previously been reported in autoimmune model [146,149]. To determine whether B cells, and particularly T2-MZP B cells had similar capacity in transplantation, I cultured FACS purified B cell subsets (T1, T2-MZP, MZ and FO B cells) with $CD4^+$ T cells for two days *in vitro*. B cells were magnetically isolated from the spleens of C57BL/6 (B6) mice (maintained in the SPF facility) and sorted by BD FACSAria into subsets (Figure 3.1 A & B). B cell subsets were co-cultured with $CD4^+$ T cells (1:1 ratio) in the presence of CD3/CD28 activator beads (1:1); plate bound anti-CD3 Abs (1μg/ml) or irradiated allo-DCs (25 T cell: 1 all-DCs) for 48 hours. Cells were incubated with PMA and Ionomycin and brefeldin A for the last 4 hours of culture. TNF-α expression was measured in $CD4^+$ T cells, and IL-10 expression in B cells, by intracellular staining and flow cytometry. Although MZ B cells, and to some extent the other B cell subsets expressed IL-10 (Figure 3.2 A & B), no B cell subset exerted any regulatory effect on T cells when they were activated with CD3/CD28 activator beads (Figure 3.3 A & B), plate bound anti-CD3 Abs (Figure 3.3 C) or allo-DCs (Figure 3.3 D). Instead there was a higher percentage of TNF-α^+ $CD4^+$ T cells in co-cultures with B cells at 48 hours compared to $CD4^+$ T cells cultured in the absence of B cells (Figure 3.3).

In summary, B cell subsets exerted no regulatory effect on activated $CD4^+$ T cells, but instead more TNF-α was expressed by T cells in the presence of B cell subsets.

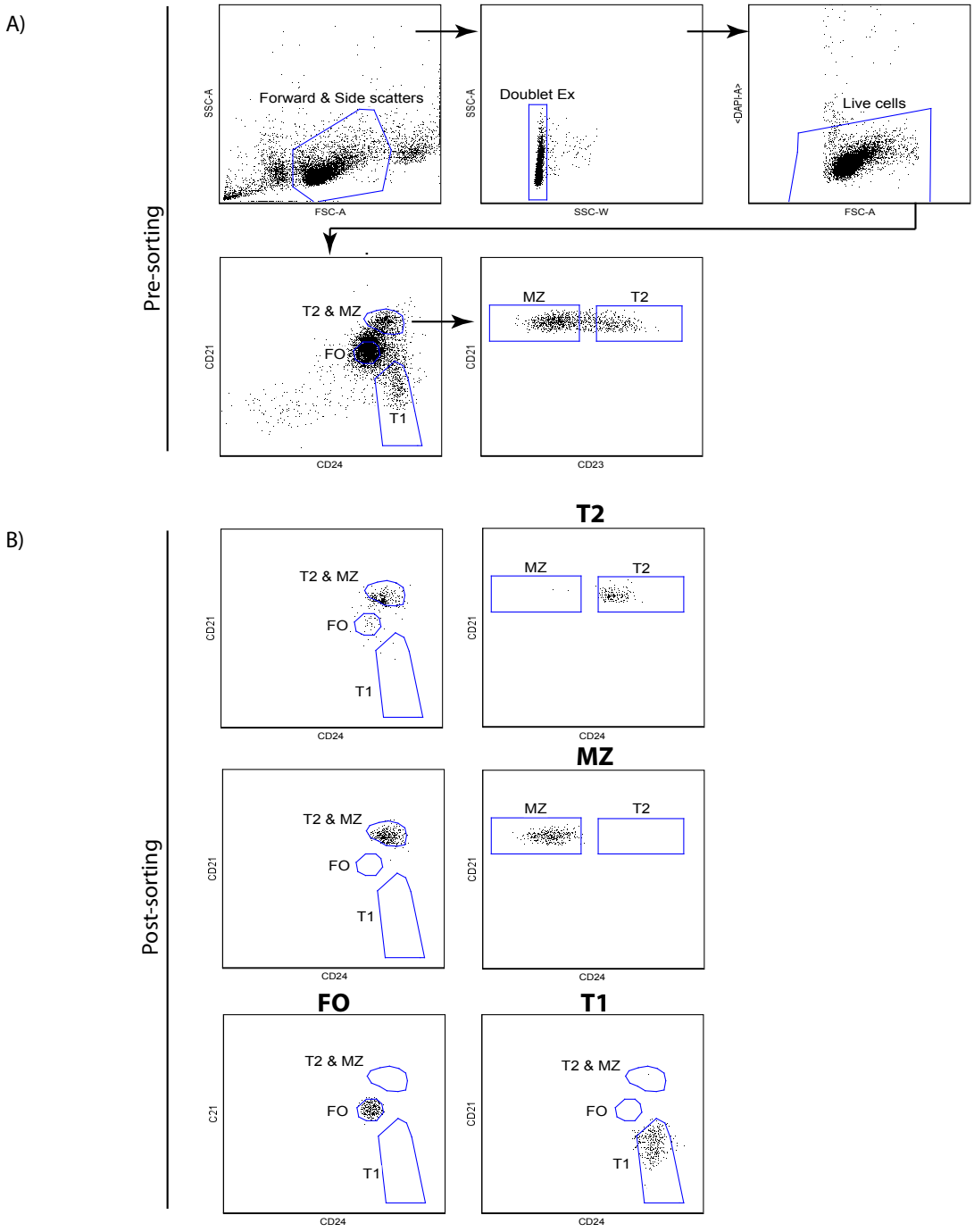

Figure 3.1 B cell subsets sorting strategies. B cells were isolated from spleens of naïve B6 mice by magnetic sorting, and stained with anti-CD21, anti-CD24, anti-CD23 and DAPI and B cell subsets were purified by BD FACSAria. FACS plots of, (A) gating strategies, (B) B cell subsets post-sorting.

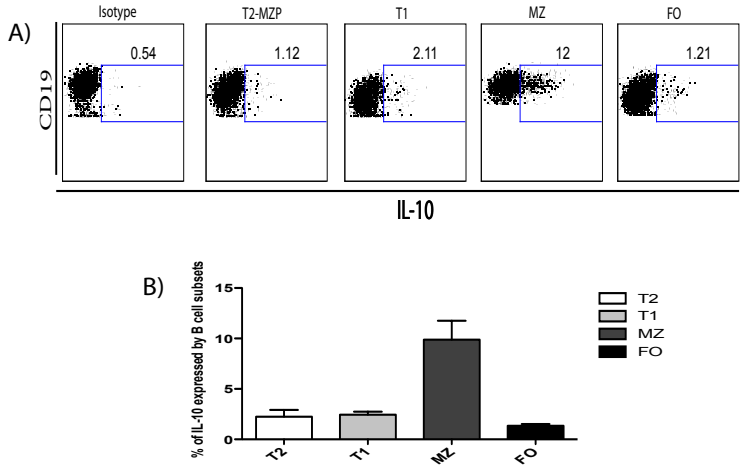

Figure 3.2 IL-10 expression by B cells isolated from B6 mice kept in the SPF facility and cultured with CD3/CD28 activated CD4$^+$ T cells . B cells were isolated from spleens of B6 mice maintained in SPF facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated CD4$^+$ T cells and CD3/CD28 activator beads (1:1) for 48 hours. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-IL-10 mAbs (ICC). (A) Representative FACS plots of CD19$^+$ B cells IL-10 expression. (B) Histogram shows mean + SEM of IL-10 expression by B cells . (n=3)

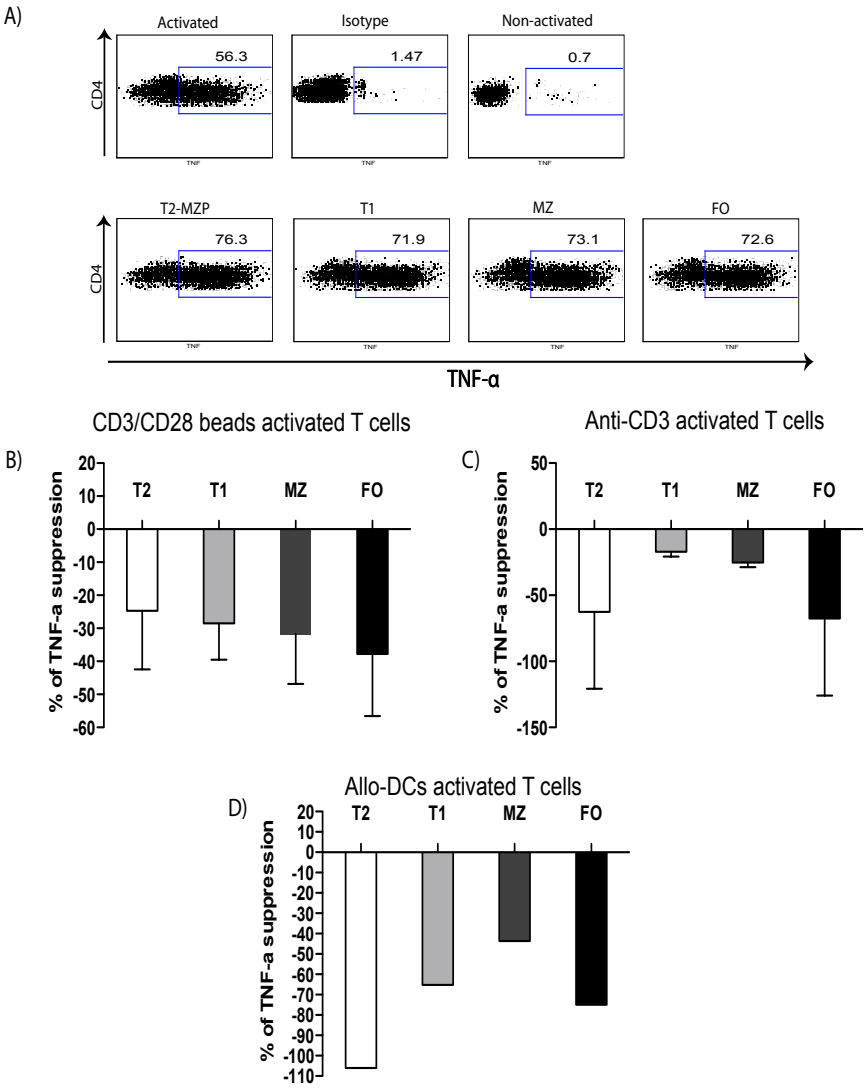

Figure 3.3 B cells isolated from SPF mice were unable to suppress CD4$^+$ T cell TNF-α production *in vitro*. B cells were isolated from spleens of B6 mice maintained in SPF facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated CD4$^+$ T cells and either CD3/CD28 activator beads (1:1), plate bound anti-CD3 (1μg/ml) or allo-DCs (25 CD4 T cells: 1 allo-DCs) for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-TNF-α mAbs (ICC). (A) Representative FACS plots of CD4$^+$ T cells TNF-α expression. (B-D) Histograms displaying mean +SEM percentage suppression of CD4$^+$ T cells TNF-α expression for, (B) CD3/CD28 beads, (C) plate bound anti-CD3, (D) Allo-DCs. n=3 for beads. n=2 for plate bound anti-CD3. n=1 for allo-DCs.

3.2 Pre-activation of B cell subsets with IL-10 promoting stimuli did not induce regulatory capacity in T2-MZP B cells.

Given that activating B cells with anti-CD40 antibody *in vitro* resulted in the recovery of IL-10 mediated Breg function in MRL/lpr mice [149], here, I addressed whether pre-activating B cells *in vitro* with stimuli shown to promote the expression of IL-10 could induce a regulatory ability in B cells in our model. After stimulating B cell subsets with combinations of LPS, CPG, LPS/CPG and anti-CD40 antibody for 48hrs, B cells showed the highest IL-10 expression when activated with LPS/CPG (representative FACS plots of IL10 expression in Figure 3.4 A). Therefore, LPS/CPG combination was selected to treat B cells before co-culture with T cells (Figure 3.4 A). FACS purified B cell subsets were cultured with CPG plus LPS for 4 hours, washed, and then co-cultured with $CD4^+$ T cells in the presence of plate bound anti-CD3 antibodies (1μg/ml) for 48 hours. Cells were incubated with PMA and Ionomycin and brefeldin A for the last 4 hours of culture. TNF-α expression was measured in $CD4^+$ T cells, and IL-10 expression in B cells, by intracellular staining and flow cytometry. Figure 3.4 B show that co-culture of CPG/LPS-activated B cells with anti-CD3-activated $CD4^+$ T cells resulted in a higher percentage of TNF-α^+ $CD4^+$ T cells than if $CD4^+$ T cells were cultured with anti-CD3 alone. Although CPG/LPS pre-activated T2-MZP B cells were unable to suppress $CD4^+$ T cell TNF-α expression, co-culture of $CD4^+$ T cells with T2-MZP B cells resulted in the lowest percentage of TNF-α^+ $CD4^+$ T cells compared to $CD4^+$ T cells cultured with the other B cell subsets (Figure 3.4.B).

In addition, I investigated whether the B cells were still expressing IL-10 after culture with anti-CD3 activated T cells. I found that after 48 hours culture with $CD4^+$ T cells a higher percentage of MZ B cells expressed IL-10 than T2-MZP cells, while IL-10 was not expressed by Transitional-1 (T1) or Follicular B cells (FO) (Figure 3.4 C). Although T2-MZP and MZ B cells produced IL-10, TNF-α expressions were higher than $CD4^+$ T cells cultured in the absence of B cell subsets. These results suggest that there is no correlation between IL-10 expressions by CPG/LPS activated B cells and their capacity to suppress $CD4^+$ T cells TNF-α expression.

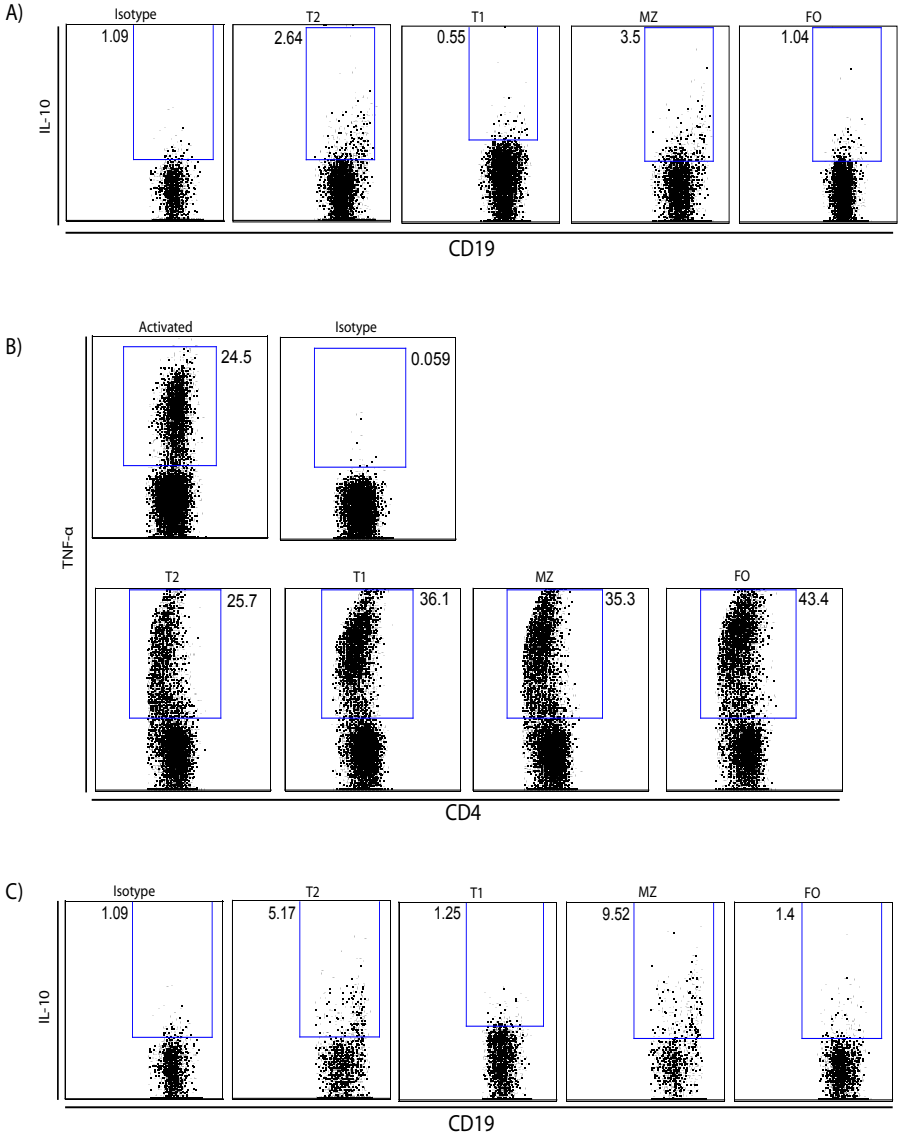

Figure 3.4 Pre-activation of B cell subsets with IL-10 promoting stimuli did not induce regulatory capacity in T2-MZP B cells. B cells were isolated from spleens of B6 mice maintained in SPF facilities by magnetic sorting. B cell subsets were purified by FACS and activated with CPG/LPS for 4hrs, washed, harvested from the wells, and co-cultured with negatively isolated $CD4^+$ T cells and plate bound anti-CD3 (1 µg/ml) for 48 hours. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-TNF-α and anti-IL-10 mAbs (ICC). Representative FACS plots of, (A) IL-10 expression by CPG/LPS activated B cell subsets prior to culture with $CD4^+$ T cells, (B) TNF-α expression by $CD4^+$ T cells cultured with B cells, (C) IL-10 expression by B cells following culture with $CD4^+$ T cells.

3.3 B cells isolated from mice maintained in CV facilities suppress TNF-α expression by $CD4^+$ T cells.

It has previously been reported that the level of sterility in which the mice are kept can influence the regulatory capacity of B cells; a subset of B cells that was suppressive in CV facilities was reported to lack this capacity in SPF facilities [274]. I thus wanted to investigate whether T2-MZP B cells would similarly recover their suppressive capacity if isolated from mice kept in less sterile conditions. To investigate whether B cells obtained from mice in the CV facility would be able to suppress TNF-α expression by $CD4^+$ T cells I isolated B cells from the spleens of CV maintained B6 mice by magnetic sorting. B cell subsets were then FACS sorted, and co-cultured with $CD4^+$ T cells in the presence of allo-DCs derived from BALB/c mice (25:1) or CD3/CD28 activator beads (1:1) for 48 hours. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. TNF-α and IL-10 were measured by intracellular staining. The results showed that T2-MZP B cells isolated from CV maintained B6 mice were able to significantly suppress allo-DCs activated $CD4^+$ T cell TNF-α expression, T1 B cells were also suppressive but the difference with the culture without B cells was not significant (Figure 3.5 A & B). T2-MZP B cells were also able to significantly suppress CD3/CD28 bead activated $CD4^+$ T cell TNF-α expression, while although MZ B cells suppressed TNF-α^+ $CD4^+$ T cells the difference with the culture without B cells was not significant (Figure 3.5 C). Within this assay B cell survival rates were measured, and the results showed that no differences between B cell subsets survival (Figure 3.5 D & E).

As with B cell - T cell co-cultures using cells isolated from SPF maintained mice

there was no correlation between IL-10 expression by B cells and their suppressive capacity when B and T cells were obtained from the CV facilities (Figures 3.5 and 3.6) Although there were significantly fewer IL-10^+ T2-MZP B cells than IL-10^+ MZ B cells in co-cultures with both allo-DCs and bead stimulated $CD4^+$ T cells, T2-MZP B cells could significantly suppress beads and allo-DCs activated $CD4^+$ T cells TNF-α expression while MZ B cells only suppressed bead activated T cells but the difference with the culture without B cells was not significant (Figure 3.6).

To summarize, there was a significant difference in the suppressive capacity of B cells isolated from CV maintained mice compared to SPF maintained mice (figure 3.7 A). B cells, in particular T2-MZP B cells, isolated from CV maintained B6 mice were able to suppress allo-DC or CD3/CD28 beads activated $CD4^+$ T cell TNF-α expression while the equivalent subsets isolated from SPF maintained mice could not. There was however no significant differences in the ability of B cells to express IL-10 between B cells isolated from mice maintained in SPF or CV facilities (Figure 3.7 B).

These results suggest that T2-MZP B cells are likely to include a regulatory B cell subset in these mice since they are the ones that suppress allo-stimulated $CD4^+$ T cells. Therefore, I sought next to determine the possible mechanism that could be involved in T2-MZP B cell regulatory function as IL-10 seems not to be correlated with T2-MZP B cells suppressive capacity.

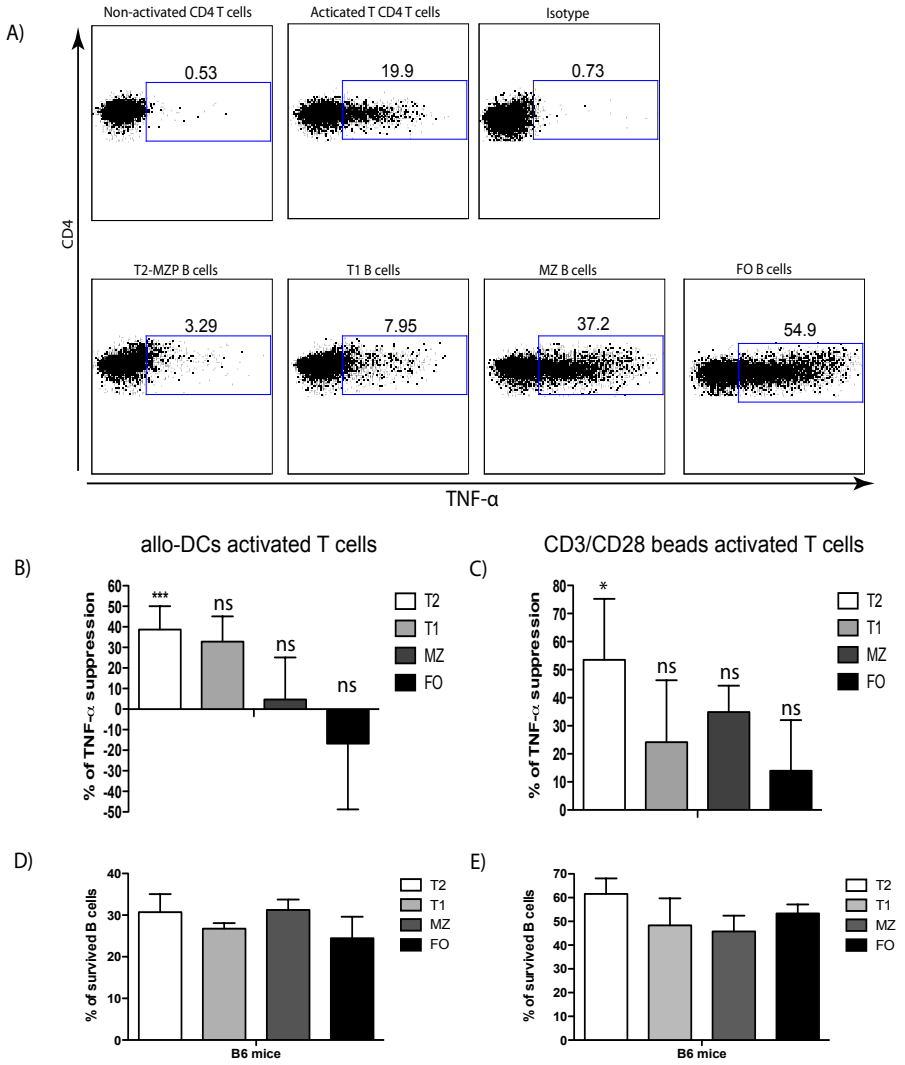

Figure 3.5 B cells isolated from mice maintained in CV facilities suppress TNF-α expression by CD4$^+$ T cells. B cells were isolated from spleens of B6 mice maintained in CV facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated CD4$^+$ T cells and either allo-DCs (25 CD4 T cells: 1 allo-DCs) or CD3/CD28 activator beads (1:1) for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-TNF-α mAbs (ICC). (A) Representative FACS plots of CD4$^+$ T cells TNF-α expression. (B-C) Histograms displaying mean± SEM percentage suppression of CD4$^+$ T cells TNF-α expression for, (B) Allo-DCs, (C) CD3/CD28 beads.n≤6. (D-E) Histograms displaying mean± SEM percentage of live B cell subsets for, (D) Allo-DCs, (E) CD3/CD28 beads.n≤6. Statistics represents difference in TNF-α expression between T cells cultured alone and with each subset. Statistics were calculated by t test or ANOVA + Dunnett's post test, *** P=0.0004 & *P<0.05.

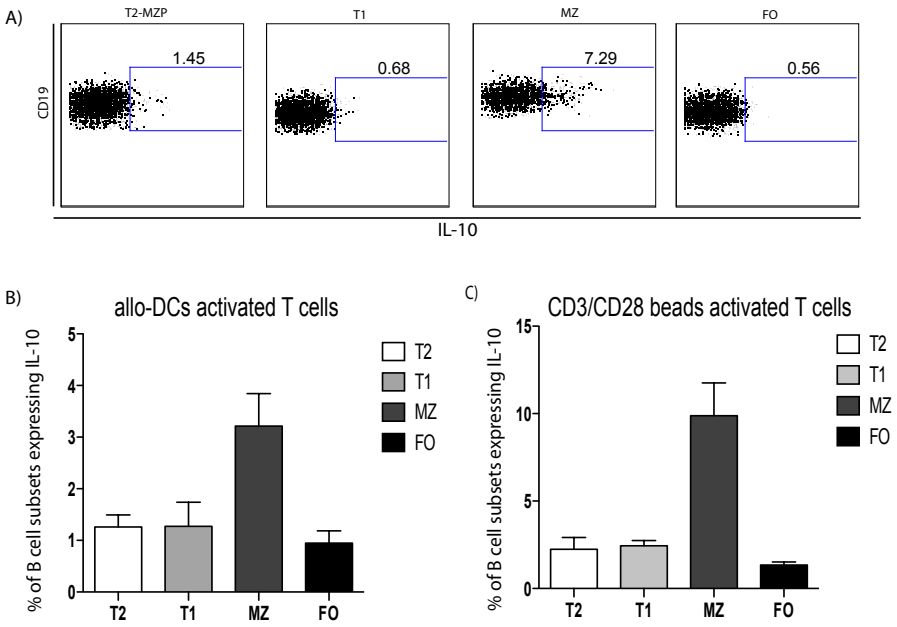

Figure 3.6 IL-10 expression by B cells isolated from B6 mice kept in the CV facilities and cultured with activated CD4$^+$ T cells. B cells were isolated from spleens of B6 mice maintained in CV facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated CD4$^+$ T cells and either allo-DCs (25 CD4 T cells: 1 allo-DCs) or CD3/CD28 activator beads (1:1) for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-IL-10 mAbs (ICC). (A) Representative FACS plots of CD19$^+$ B cells IL-10 expression. (B-C) Histograms displaying mean± SEM percentage of B cells IL-10 expression, (B) Allo-DCs, (C) CD3/CD28 beads. n=3.

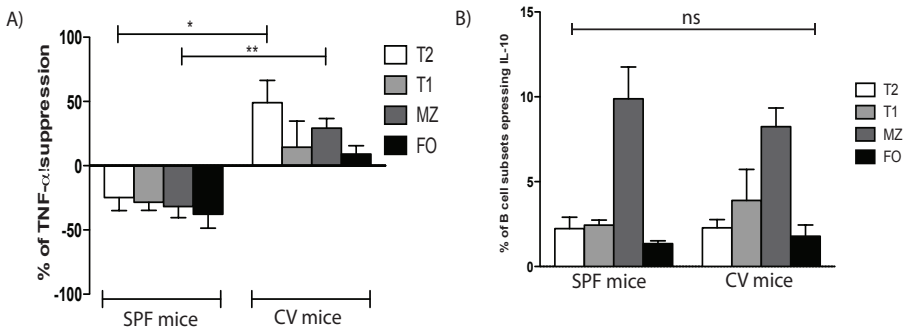

Figure 3.7 T2-MZP & MZ B cells isolated from mice kept in CV facilities suppressed T cells activation significantly better than the same subsets isolated from mice kept in SPF facilities. B cells were isolated from spleens of B6 mice maintained in CV & SPF facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated $CD4^+$ T cells and CD3/CD28 activator beads (1:1) for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. (A-B) Histograms shows mean±SEM of, (A) the percentage of TNF-α inhibition, (B) the percentage of IL-10 expression by B cells. n=3. Statistics represents difference in TNF-α inhibition between T cells cultured with subsets isolated from mice kept in CV and SPF facilities. Statistics were calculated by t test, ** P=0.0059 & *P=0.0184.

3.4 The suppressive capacity of T2-MZP B cells requires the expression of Gal-1 and CD80/CD86 molecules.

Gal-1 has been shown to have an immunoregulatory role in the immune responses [187,190,191]. Therefore, I sought to determine whether Gal-1 plays a role in T2-MZP B cell suppressive capacity. First, to confirm whether B cells express Gal-1, I initially examined its expression in total B cells by western blot. B cells were isolated from three naive B6 mice by magnetic sorting, and the cells ($5x10^5$ cells) were then lysed and the lysates used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Subsequently, the gel was passed through the steps that have been described previously in the methods for western blot preparation. As shown in Figure 3.8, B cells express Gal-1.

To examine the difference in the ability of B cells isolated from Gal-$1^{-/-}$ mice kept in the SPF and CV facilities to express IL-10. B cells were isolated from Gal-$1^{-/-}$ and B6 (as a control) mice housed in the SPF and CV facilities and stimulated with various stimuli (LPS, CPG and anti-CD40 mAb) for 48 hours. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture. IL-10 expression was measured in B cells, by intracellular staining and flow cytometry. These primary results showed that B cells isolated from Gal-$1^{-/-}$ mice kept in the SPF and CV facilities expressed IL-10 at the same levels (Figure 3.9 A).

Then, to examine Gal-1 role in T2-MZP B cell suppressive function, splenic B cells were magnetically isolated from B6 and Gal-$1^{-/-}$ mice housed in the CV facilities. Subsequently, B cell subsets were FACS sorted, and co-cultured with

allo-DCs (irradiated) and $CD4^+$ T cells (isolated from naïve B6 mice) for 48 hours. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture. TNF-α expression was measured in $CD4^+$ T cells, and IL-10 expression in B cells, by intracellular staining and flow cytometry. T2-MZP B cells isolated from Gal-$1^{-/-}$ mice lost the ability to suppress $CD4^+$ T cells TNF-α expression compared to B cells from B6 mice (Figure 3.9 B and C). Again, there was no significant difference in the ability of B cell subsets between the two strains of mice to express IL-10 (Figure 3.9 D). These results suggest that the capacity of T2-MZP B cells to suppress allo-DCs activated T cells is Gal-1 dependent and does not correlate with the capacity of B cells to express IL-10.

In addition, it has been reported that B cells provide essential signals for the activation and proliferation of autoreactive $CD4^+$ T cells via CD80 and CD86 mediated interactions [136]. To examine the role of CD80 and CD86 molecules, B cells were isolated from B6 mice housed in CV facilities by magnetic sorting and the B cell subsets were FACS sorted. The B cell subsets were co-cultured with $CD4^+$ T cells stimulated by CD3/CD28 beads with and without anti-CD80 and anti-CD86 neutralizing antibodies (5μg/ml) for 48 hours. Subsequently, PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture. TNF-α expression was measured in $CD4^+$ T cells by intracellular staining and flow cytometry. I found that T2-MZP B cells lost their suppressive ability in the presence of anti-CD80 and anti-CD86 neutralizing Abs (Figure 3.10 A), whereas neutralizing CD80 and CD86 molecules did not affect the suppressive function of MZ B cells (Figure 3.10 B). This result demonstrates that T2-MZP B cells require CD80 and CD86 molecules to exert their regulatory effect.

In summary, the inhibitory effect of T2-MZP B cells depends on the presence of Gal-1 and on cell contact through CD80 and CD86. However the suppressive capacity of MZ B cells on CD3/CD28 bead activated $CD4^+$ T cells seems not to be associated with CD80/CD86 molecules.

Moreover, our attempt to inhibit IL-10 and the extracellular Gal-1 by adding anti-IL-10 and anti-IL-10 receptors neutralizing antibodies and lactose (to block Gal-1) to our suppressive assay has failed. One of the reasons was the direct effect of the antibodies and the lactose on T cell activation. As shown in Figure 3.11 most of the reagents augmented per se the production of TNF-α by T cells.

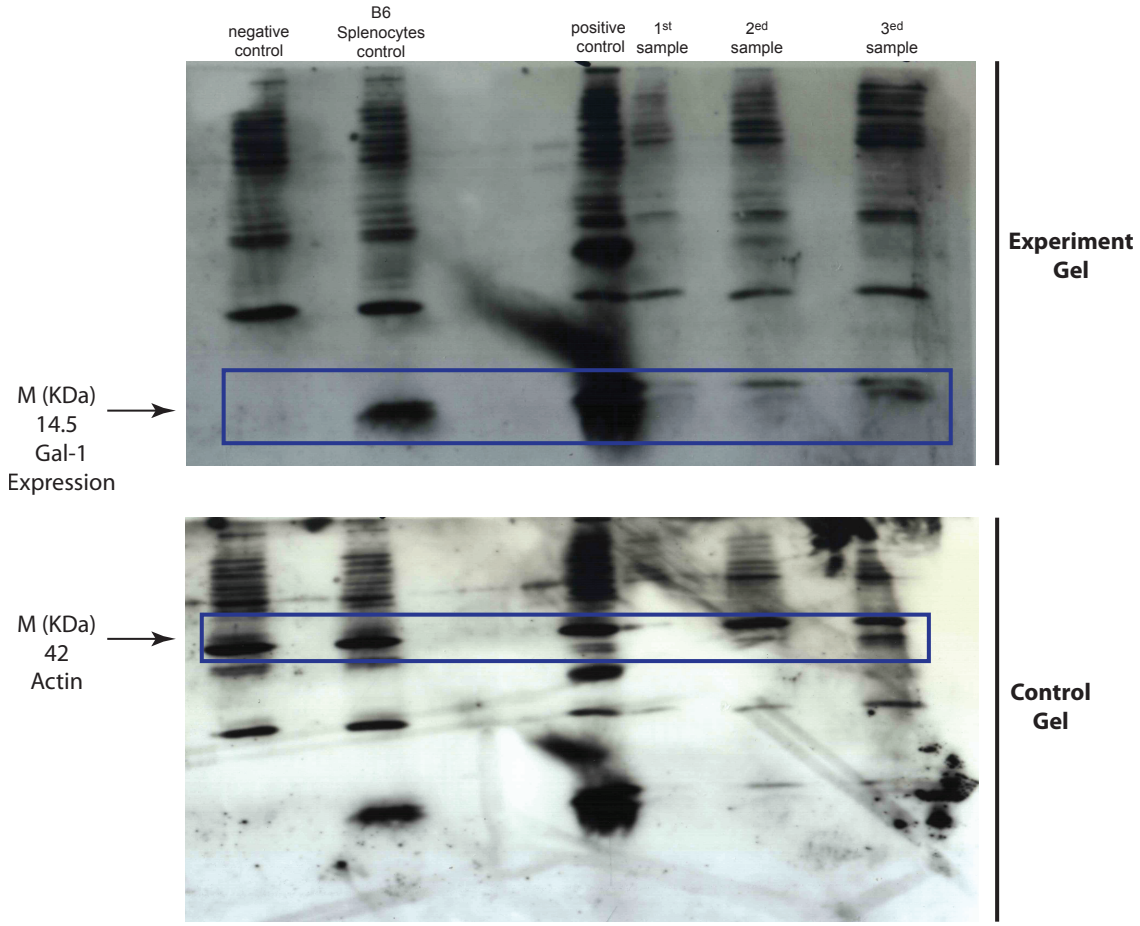

Figure 3.8 B cells express Gal-1. B cells were isolated from spleens of naïve B6 mice by magnetic sorting, lysed ($5x10^5$ cells) and then were run overnight through SDS-PAGE. The proteins were then transferred onto immobilon-P transfer membrane, using a semidry transfer system. The membranes were subsequently probed with mouse anti-human gal-1 mAb. Gels were then incubated with goat HRP-conjugated rabbit anti-mouse IgG, and developed with ECL plus western blotting detection reagent. Regulatory T cells were obtained from a Treg line, and used as a positive control. Gal-$1^{-/-}$ B cells were used as negative control (n=3 B6 mice).

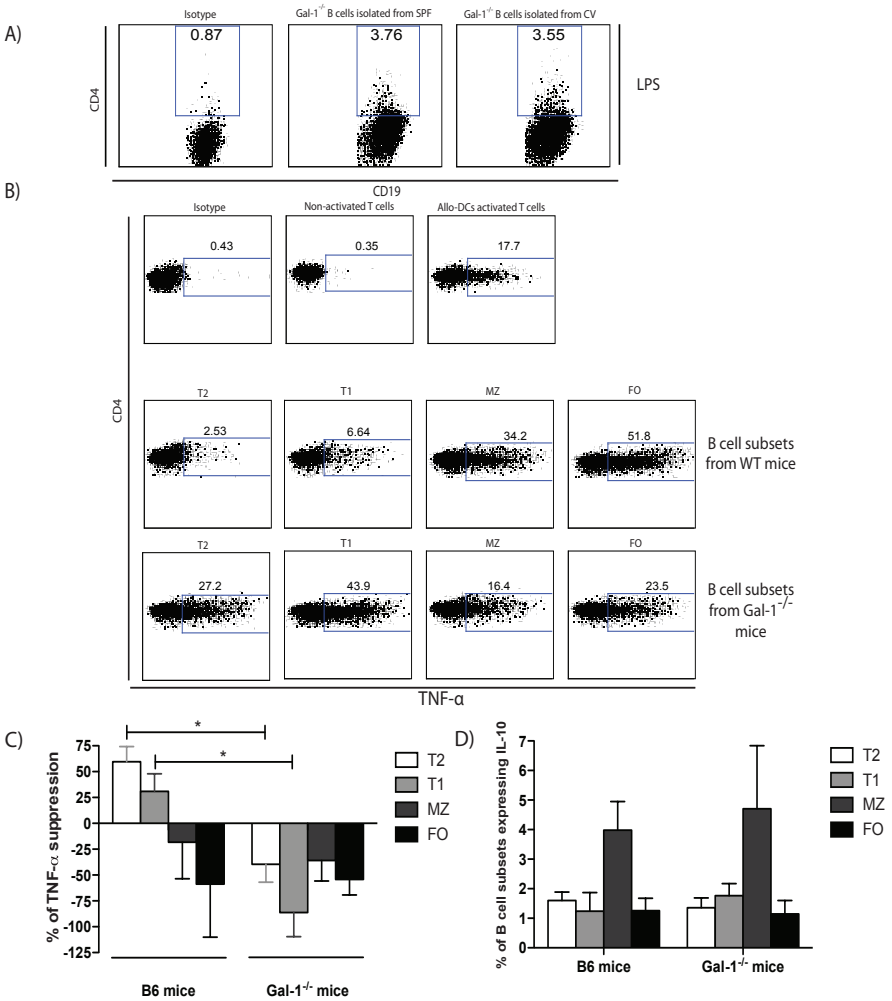

Figure 3.9 Gal-1 expression is essential for T2-MZP and T1 B cell suppressive function. B cells were isolated from spleens of B6 and Gal-$1^{-/-}$ mice maintained in CV and SPF facilities by magnetic sorting, and stimulated with various stimuli (LPS, CPG and anti-CD40 Ab) for 48 hours. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture. (A) Representative FACS plots of LPS stimulated CD19 B cells IL-10 expression. (B,C & D) B cells were isolated from spleens of B6 and Gal-$1^{-/-}$ mice maintained in CV facilities by magnetic sorting. B cell subsets were purified by FACS and co-cultured with negatively isolated $CD4^+$ T cells and allo-DCs (25 CD4 T cells: 1 allo-DCs) for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-TNF-α and anti-IL-10 mAbs (ICC). (B) Representative FACS plots of $CD4^+$ T cells TNF-α expression. (C-D) Histograms shows mean± SEM of , (B) the percentage of TNF-α inhibition, (B) the percentage of IL-10 expression by B cells. n=3. Statistics represents difference in TNF-α inhibition between T cells cultured with subsets isolated from Gal-$1^{-/-}$ and B6 mice kept in CV facilities. Statistics were calculated by t test, *P<0.05.

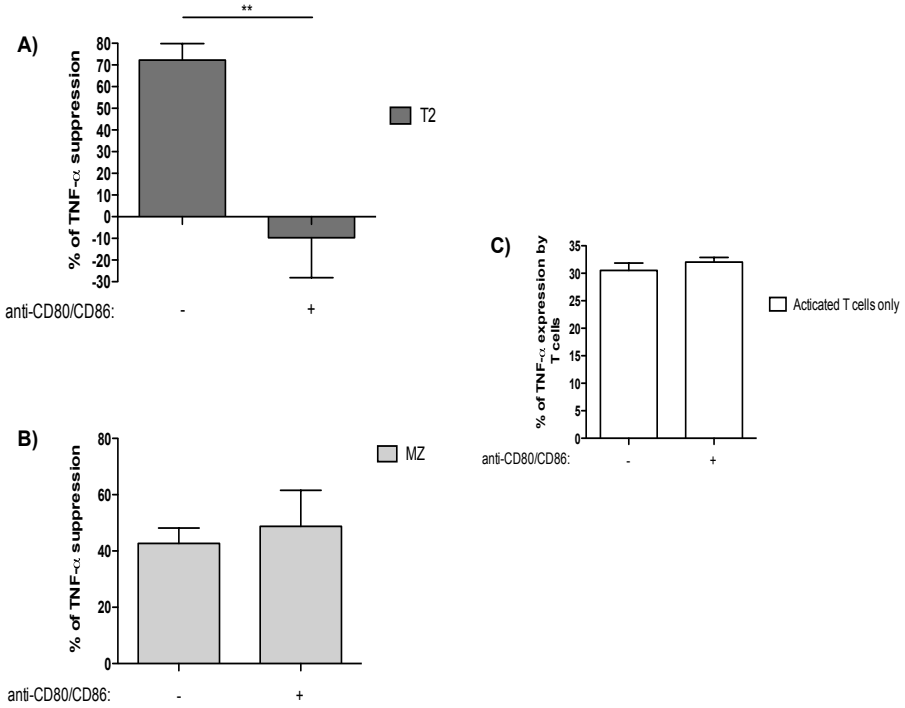

Figure 3.10 The suppressive capacity of T2 B cells requires the expression of Galectin-1 and CD80/CD86. B cells were isolated from spleens of B6 mice maintained in CV facilities by magnetic sorting. T2 & MZ B cells were purified by FACS and co-cultured with negatively isolated $CD4^+$ T cells and activator CD3/CD28 beads (1:1) with or witout anti-CD80 and anti-CD86 Abs for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD19 and anti-CD4 mAbs (surface), and anti-TNF-α mAbs (ICC). (A-C) Histograms shows mean± SEM of , (A) the percentage of TNF-α inhibition with and without anti-CD80/CD86 mAbs, and in the presence of T2 B cells, (B) the percentage of TNF-α inhibition with and without anti-CD80/CD86 mAbs, and in the presence of MZ B cells, (C) CD3/CD28 activated T cells with and without anti-CD80/CD86 mAbs. n=3. Statistics were calculated by t test, ** P=0.0074.

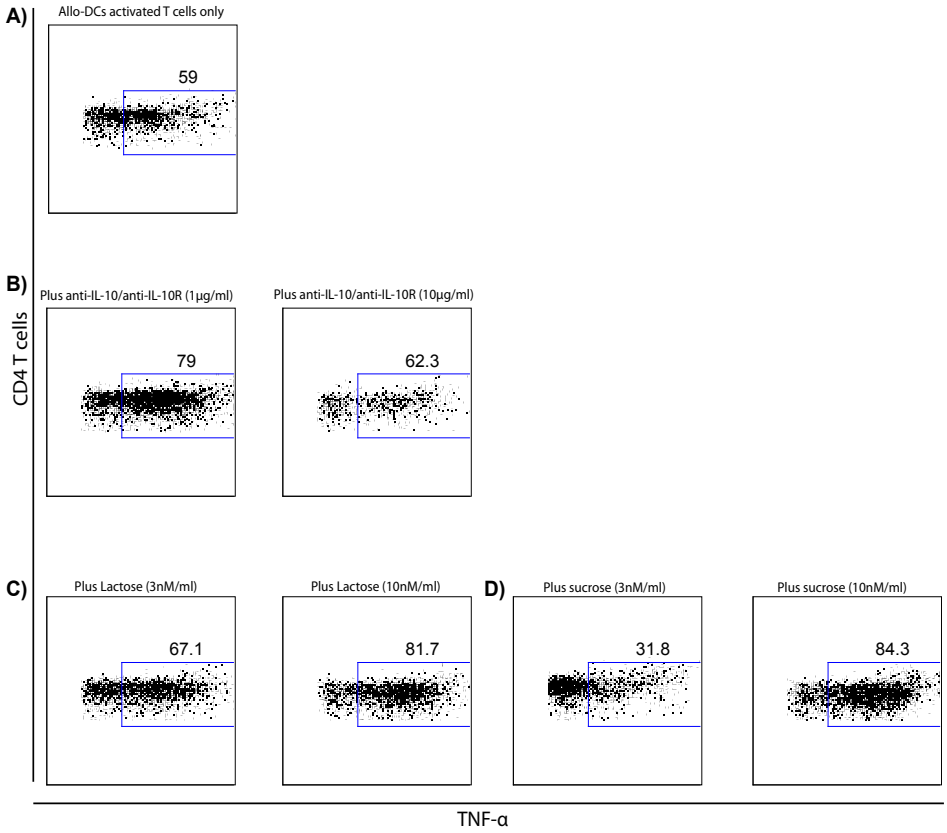

Figure 3.11 The suppressive capacity of T2-MZP B cells requires the expression of Galectin-1 and CD80/CD86. $CD4^+$ T cells were negatively isolated from spleens of B6 mice maintained in CV facilities by magnetic sorting and co-cultured with allo-DCs (25 CD4 T cells: 1 allo-DCs) with or without anti-IL-10 and anti-IL10 receptors, or lactose and sucrose for 48hrs. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. Subsequently, cells were incubated with anti-CD4 mAbs (surface), and anti-TNF-α mAbs (ICC). Representative FACS plots of $CD4^+$ T cells TNF- α expression co-cultured with allo-DCs, (A) alone, (B) with anti-IL-10 and anti-IL10 receptors at different concentrations, (C) with lactose and (D) sucrose at different concentrations. n=1.

3.5 T2-MZP and T1 B cells isolated from mice kept in the CV facilities can prolong MHC I mismatched skin graft survival.

The adoptive transfer of T2-MZP B cells has previously been reported to suppress the development of several murine experimental models of autoimmunity. For instance, adoptive transfer of T2-MZP B cells suppressed CIA and Lupus [146, 149]. Moreover, a recent report in a transplantation model showed that the transfer of splenic TIM-1^+ B cells, but not TIM-1^- B cells, which were isolated from anti-TIM-1 treated islet transplant recipient mice 14 days post transplant, were able to prolong the survival of islets following adoptive transfer to B cell deficient JHD mice [173]. Therefore, I sought to determine if B cells, in particular T2-MZP B cells had similar suppressive capacity in our model. Our *in vitro* data suggested that B cells from the SPF facility would not be able to prolong graft survival but that B cells from the CV facilities might. To investigate this possibility, T1, T2-MZP, FO and MZ B cells were FACS sorted from the spleens of naïve B6 mice maintained in SPF facilities and $1x10^6$ cells (maximum number of T2-MZP B cells acquired after sorting) of each subset were adoptively transferred (IV) to naïve B6 mice. The day after B6 mice (H-2^b) received 1cmx1cm dorsal grafts of tail skin from MHC I mismatched transgenic B6 mice that express H2-K^d (B6-K^d). $CD8^+$ T cells were depleted by IP injection of anti-CD8 antibody on day -1, 0 (day of skin graft), +1 and every 7 days following the skin graft. Grafts were said to be rejected following the formation of a hard black eschar (Figure 3.12). The results in Figure 3.13 show that the adoptive transfer of B cell subsets isolated from SPF maintained mice did not prolong skin graft survival compared to control mice for any of the transferred

subsets, confirming our hypothesis about SPF B cells.

Given that the *in vitro* data suggested that maintenance of mice in CV facilities, as opposed to SPF facilities, allows the isolation of functional regulatory B cells, I repeated these experiments using mice housed in CV facilities. As skin transplant experiments are usually carried out in SPF facilities I first examined the feasibility of conducting skin grafts in CV facilities given the higher risk of skin infection, which could result in unexpected rejection. Therefore, I performed skin transplants on two groups of B6 mice. One group received B6 K^d skin and one received B6 skin, and skin survival was monitored for 100 days. I found that B6 K^d skin recipients rejected within 8 to 9 days (earlier than the rejections observed with B6 K^d skin recipients maintained in SPF facilities (13 days)), while B6 skin recipients had still not rejected the skin graft by day 100 (Figure 3.14 A). These results suggested that the greater risk of infection was not a barrier to performing skin transplantation experiments in CV facilities.

Given that, I repeated the adoptive transfer experiments I had carried out in the SPF facilities in the CV facilities. B6 mice received injections of 1 $x10^6$ T1, T2-MZP, MZ or FO B cell subsets one day before skin transplant as above. In addition, I compared the effects of injecting B cells isolated from B6 or Gal-$1^{-/-}$ mice (controls were injected with PBS). As above recipient B6 mice were depleted of $CD8^+$ T cells by injections of anti-CD8 at day's -1, 0 (day of skin graft), +1 and every 7 days following the skin graft. To exclude rejection due to infection, I added as control a group of B6 mice transplanted with B6 skin in every experiment performed. In agreement with our *in vitro* results, T2-MZP and, T1 B cells significantly prolonged the survival of B6-K^d skin graft compared

to control mice mean graft survivals were 15 days, 13 days, and 10 days for T2-MZP, T1 and controls respectively. Although transfer of MZ B cells significantly prolonged skin survival (mean survival 12 days) compare to the control, adoptive transfer of T2-MZP B cells resulted in significantly longer graft survival compared to MZ B cells. In contrast, FO B cells did not prolong allograft survival (mean 10) (Figure 3.14 B). Moreover, the involvement of Gal-1 in T2-MZP B cells regulatory function was confirmed in this *in vivo* experiment. Adoptive transfer of Gal-$1^{-/-}$ T2-MZP, T1 or MZ B cells did not prolong B6 K^d skin survival compared to controls. Recipients that received Gal-$1^{-/-}$ T2-MZP B cells rejected the graft (mean survival 10 days) at the same time as the control group (mean survival 15 days) (Figure 3.14 B). Although the absence of Gal-1 has affected T1 and MZ B cells to lose their capacity to prolong skin graft survival, it was not significant compared to the WT T1 and MZ B cells (mean survival 9 & 10, respectively).

In addition, serum was collected from these mice at 30days post-transplant (from both SPF and CV experiments) in order to evaluate any differences in allo-specific IgG levels in these animals. There was no significant difference in the alloantibody levels in the serum between mice receiving the different B cell subsets (Figure 3.13 B and 3.14 D).

Collectively, T2-MZP, T1 and MZ B cells prolonged graft survival, however, T2-MZP B cells provided significantly better survival compared to MZ B cells (look at the discussion for T1). These B cell subsets lost their capacity to prolong skin graft survival when isolated form Gal-$1^{-/-}$ mice.

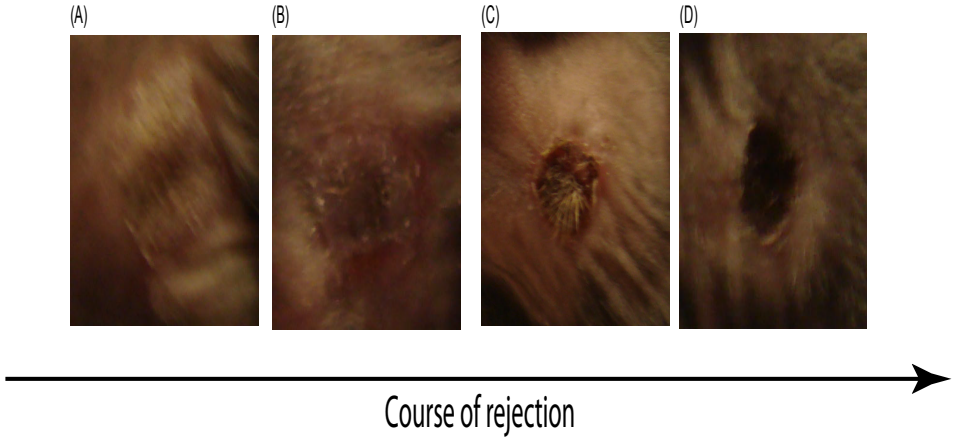

Figure 3.12 Representative pictures of the time course of skin rejection. CD8 depleted B6 mice received 1cmx1cm tail skin graft from B6 K^d mice. Pictures shows, (A) healthy skin immediately after cast removal, (B) as graft begin to reject, (C) 80% rejection, (D) total rejection with hard black eschar.

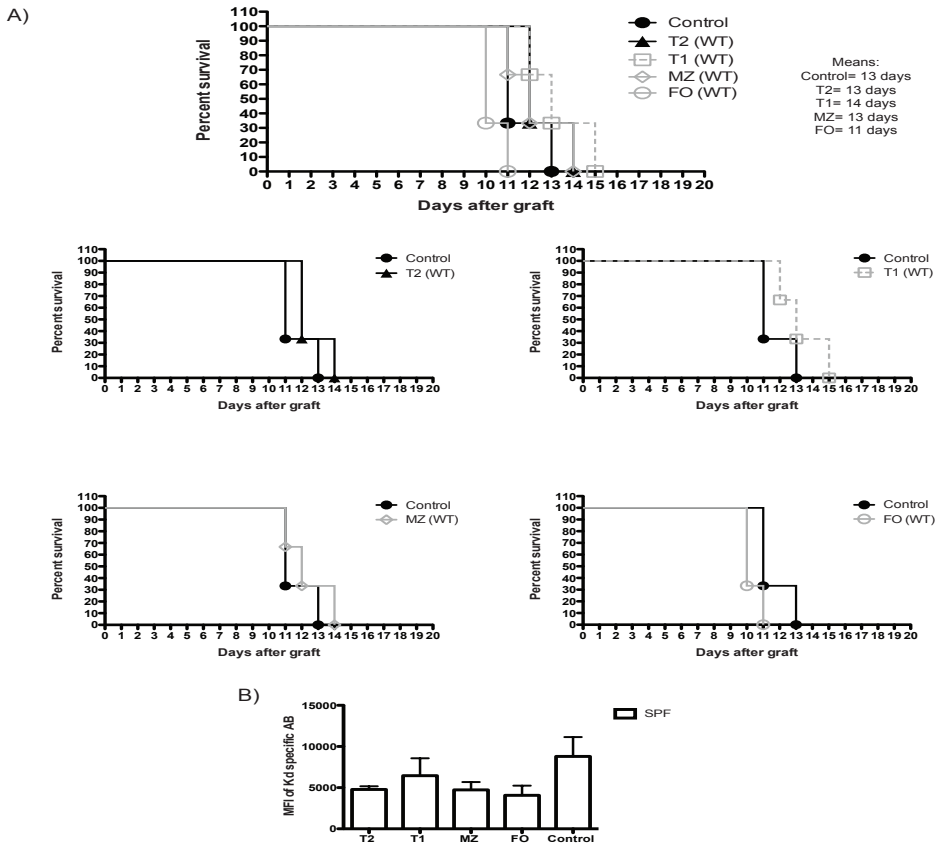

Figure 3.13 Adoptive transfer of T2-MZP and T1 B cells isolated from mice kept in SPF facilities can prolong MHC I mismatched skin graft survival. CD8 depleted B6 (H2-K^b) mice received dorsal skin grafts from B6 K^d (H2-K^d) mice. One day prior to skin graft mice received $1x10^6$ T2-MZP, T1 , FO, MZ B cells that were FACS purified from naïve B6 mice, or PBS. Mice were monitored daily for skin rejection and grafts were said to be rejected when they formed hard black eschars (Figure 3.11). (A) B6-K^d skin graft survival on B6 recipients of B6 or Gal-$1^{-/-}$ B cell subsets. (B) Mean ± SEM of anti-K^d antibody production in the serum of B6 mice that received K^d skin grafts and B6 B cell subsets at day 30-post graft. n=3/group for each experiment. Statistics were calculated by log-rank test.

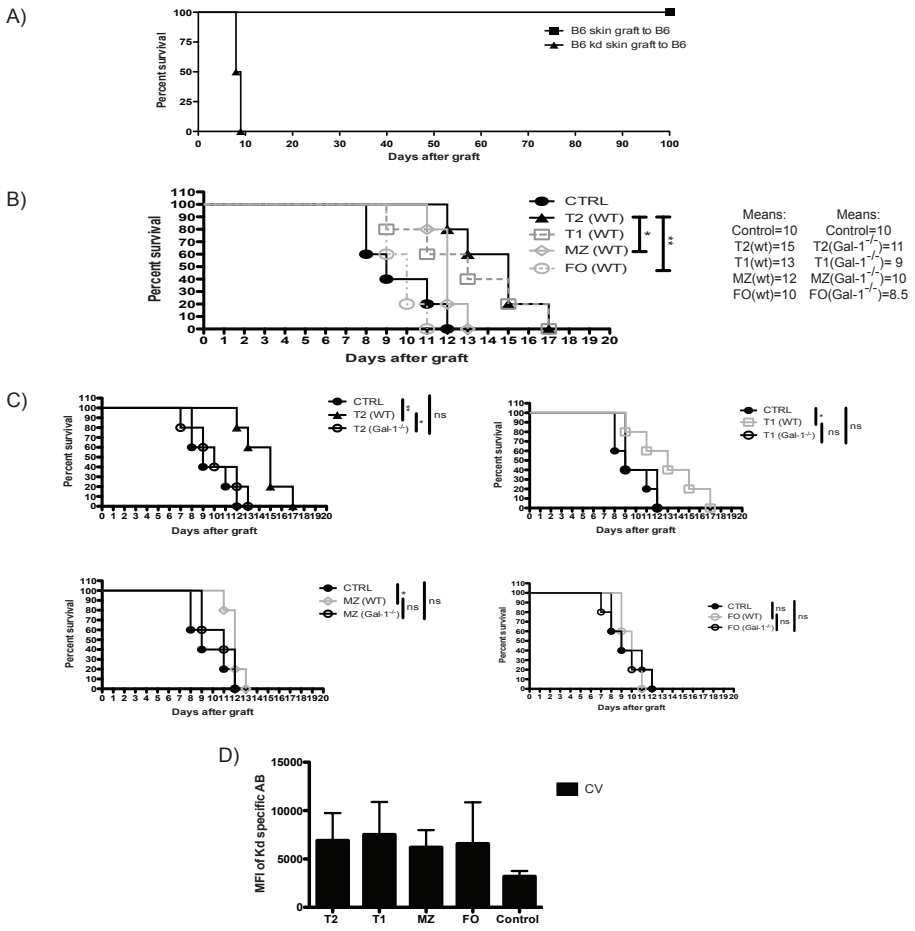

Figure 3.14 Adoptive transfer of T2-MPZ and T1 B cells isolated from mice kept in CV facilities can prolong MHC I mismatched skin graft survival. CD8 depleted B6 (H2-K^b) mice received dorsal skin grafts from B6 K^d (H2-K^d) mice, or B6 mice as infection controls. one day prior to skin graft mice received $1x10^6$ T2-MZP, T1 , FO, MZ B cells that were FACS purified from naïve B6 or Gal-$1^{-/-}$ mice, or PBS. Mice were monitored daily for rejection and grafts were said to be rejected when they formed hard black eschars (Figure 3.12). (A) B6 or B6 K^d skin graft survival on B6 recipients. (B) K^d skin graft survival on B6 recipients of WT B cell subsets. (C) B6-K^d skin graft survival on B6 recipients of B6 or Gal-$1^{-/-}$ B cell subsets. (D) Mean ± SEM of anti-K^d antibody production in the serum of B6 mice that received K^d skin grafts and B6 B cell subsets at day 30-post graft. n≥3/group for each experiment. Statistics were calculated by log-rank test. *P<0.05 & **P<0.005.

3.6 No differences in B cell subset proportions, but significant differences in the percentages of memory $CD4^+$ & $CD8^+$ T cells in mice maintained in CV & SPF facilities.

Next, I wanted to determine if there were obvious immunological differences between mice kept in CV and SPF facilities that might have explained the difference in the capacity of B cells to prolong skin graft survival. First I addressed whether the mice being kept in SPF or CV facilities influenced B and T cell subsets proportions. Spleens and lymph nodes were collected from mice in both facilities, and the cells were incubated with anti-CD19, anti-CD21, anti-CD23 and anti-CD24 Abs for flow analysis of B cell subsets. To investigate any difference in T cell subsets, I stained the lymphocytes and splenocytes with anti-CD8, anti-CD4, anti-CD25, anti-CD44 and anti-CD62L Abs to identify memory and naïve $CD4^+$ and $CD8^+$ T cells. The results showed no differences in the proportions of the various B cell subsets in the spleens and lymph nodes of mice maintained in SFP and CV facilities (Figure 3.15 A). On the other hand, there were significantly higher percentages of total $CD8^+$ T cells in the spleens of B6 mice maintained in SPF facilities compared to mice maintained in CV facilities (Figure 3.15 B & C). In addition, in the lymph nodes, CV mice expressed significantly higher percentages of total $CD4^+$, memory $CD4^+$ and $CD8^+$ T cells (Figure 3.15 C & E). These results suggest that T cells have been primed *in vivo* in mice kept in the CV facilities.

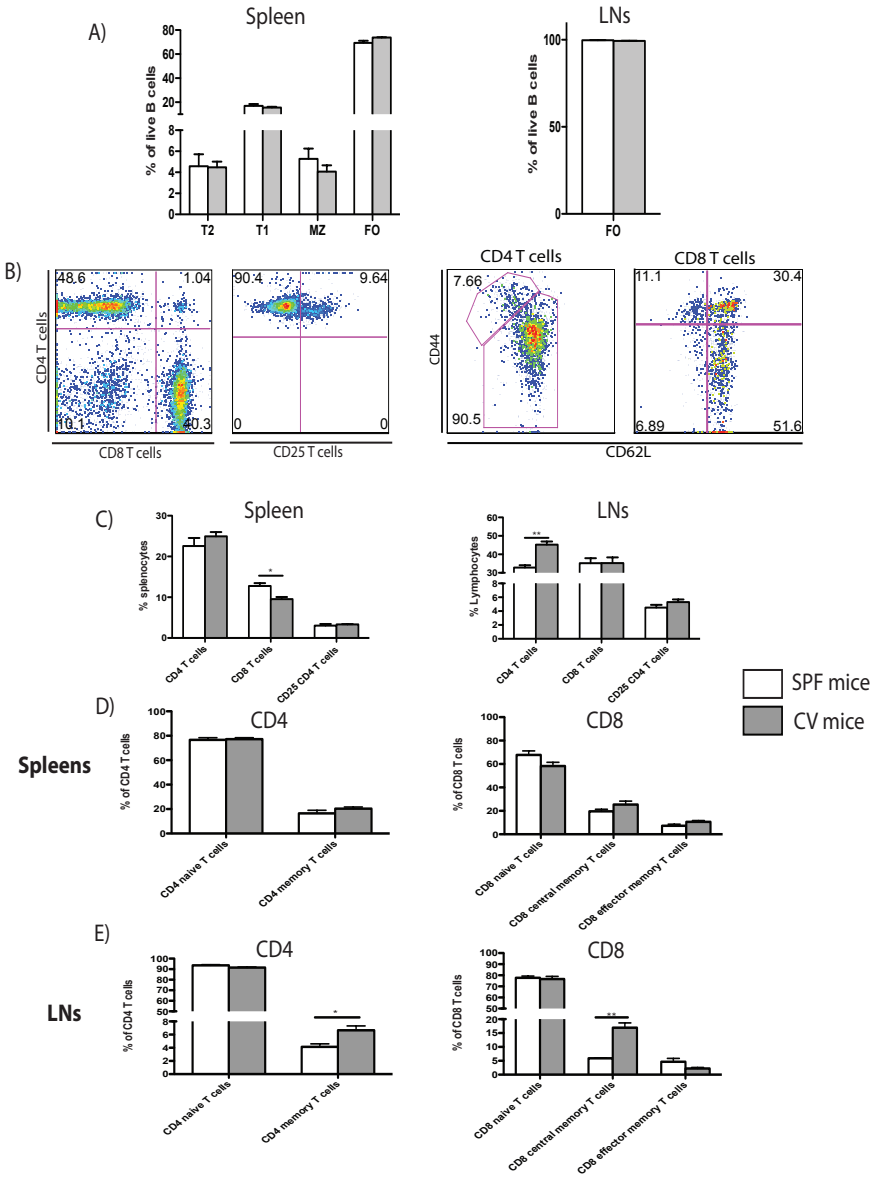

Figure 3.15 Increase percentages of memory CD4$^+$ and CD8$^+$ T cells in LNs of mice kept in CV facilities compared to mice kept in SPF facilities. Spleens and LNs were collected from B6 mice (6 weeks old) that had been kept in the SPF and the CV facilities, and phenotyped for B cell and T cell populations by using anti-CD4, anti-CD8, anti-CD4, anti-CD25, anti-CD19, anti-CD21, anti-CD23, anti-CD24, anti-CD44, & anti-CD62L mAbs. (A) Histograms displaying mean ± SEM of a comparison of B cell subsets in the spleens (left) and LNs (right) from SPF and CV mice. (B) Representative FACS plots of T cells gating strategies. Histograms shows mean ± SEM of a, (C) Comparison between CD4$^+$, CD8$^+$ and CD4$^+$CD25$^+$ in the spleens (left) and LNs (right) from SPF and CV mice, (D) Comparison between CD4$^+$ (left) and CD8$^+$ (right) T cell populations in the spleens of SPF and CV mice, (E) Comparison between CD4$^+$ (left) and CD8$^+$ (right) T cell populations in the LNs of SPF and CV mice. n=3. Statistics were calculated by t test, *P<0.05 & **P<0.005.

3.7 T & B cells isolated from mice maintained in CV facilities express higher levels of co-stimulatory molecules compared to mice kept in SPF facilities.

I next evaluated the expression of co-stimulatory molecules on $CD8^+$, $CD4^+$ and $CD19^+$ cells, in the spleens and lymph nodes of mice that have been kept in the CV and SPF facilities. In the spleens, $CD4^+$ and $CD8^+$ T cells expressed marginal but significantly higher levels of CD80 molecule when isolated from mice that have been kept in CV facilities compared to those maintained in SPF facilities (Figure 3.16 A & B). The differences were small however and whether they are physiologically relevant is unclear. In addition, there was a trend for a lower level of expression of CD80 molecule on the same cells, although this was not significant (Figure 3.16 A & B). $CD4^+$ and $CD8^+$ T cells isolated from lymph nodes of mice maintained in CV facilities expressed significantly higher levels of co-stimulatory molecules compared to mice maintained in SPF facilities, but again whether they are physiologically relevant is unclear (Figure 3.16 C). B cells expressed significantly higher levels of MHC I and II in lymphocytes isolated from mice that have been kept in CV facilities compared to cells obtained from mice maintained in SPF facilities (Figure 3.16 D).

These results suggest that cells from mice maintained in CV facilities are more activated and primed than cells from mice maintained in SPF facilities.

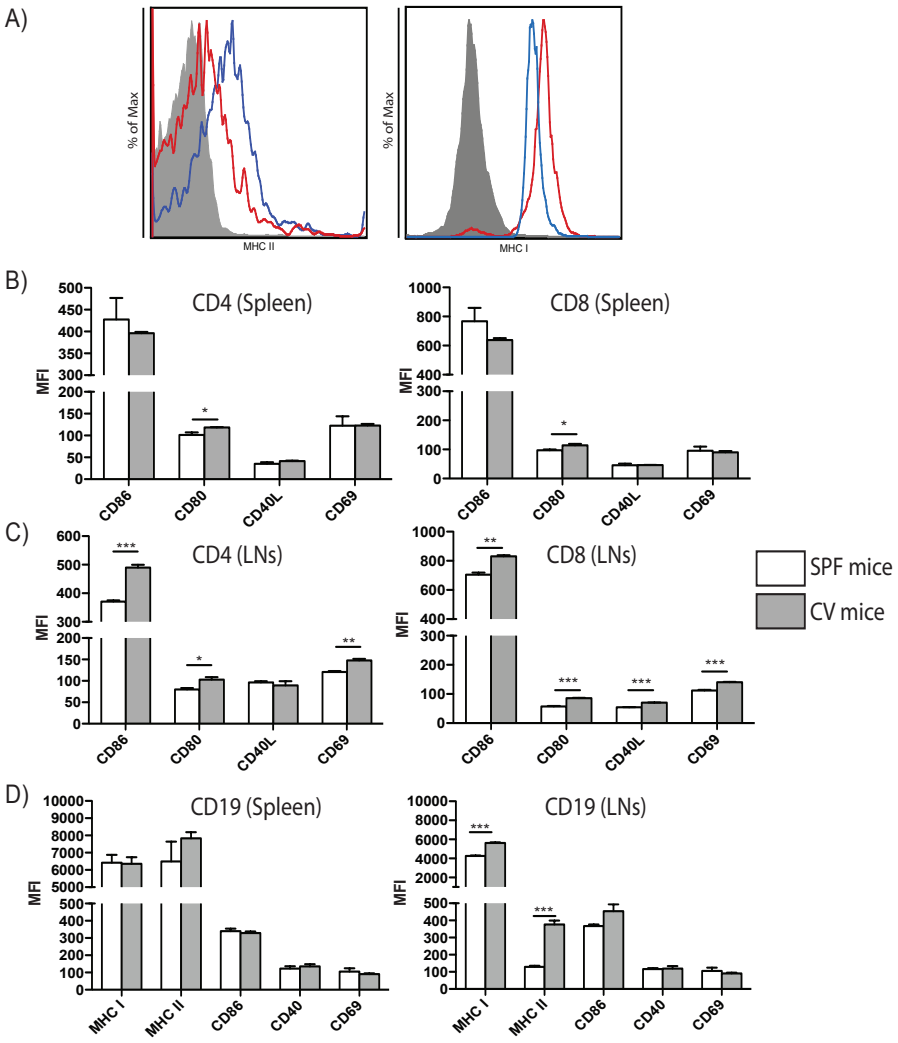

Figure 3.16 Expression of co-stimulatory molecules on B and T cells isolated from mice kept in SPF and CV facilities. Spleens and LNs were collected from mice (6 weeks old) that have been kept on the SPF and the CV facilities, and phenotyped with anti-CD4, anti-CD8, anti-CD19, anti-MHC I, anti-MHC II, anti-CD80, anti-CD86, anti-CD40, anti-CD40L and anti-CD69 Abs. (A) Representative histograms of MHC II (left) and MHC I expression (right) on $CD19^+$ B cells obtained from mice kept in the SPF (red) and in the CV (blue) facilities, isotype controls are shown as solid grey histograms. Histograms displaying the MFI, (B) expression of co-stimulatory molecules on $CD4^+$ (right) and $CD8^+$ T cells (left) obtained from the spleens of mice kept in the SPF or in the CV facilities, (C) expression of co-stimulatory molecules on $CD4^+$ (right) and $CD8^+$ T cells (left) obtained from the LNs of mice kept in the SPF or in the CV facilities, (D) expression of co-stimulatory molecules on $CD19^+$ B cells obtained from spleens (left) and the LNs (right) of mice kept in the SPF or in the CV facilities. Graphs show mean± SEM, n=3. Statistics were calculated by t test, * P<0.05, **P<0.005 & ***P<0.0005.

3.8 Differences in the response to *in vitro* activation of splenocytes isolated from mice kept in SPF and CV facilities.

I next sought to determine whether B and T cells isolated from mice maintained in CV and SPF facilities responded differently upon activation. In this experiment we activated splenocytes obtained from mice maintained in CV and SFP facilities with PMA and Ionomycin for 5 hours. CD69, CD86, MHC I, and MHC II molecules were measured on B and T cells. In addition the expression of IFN-γ, IL-10 and TNF-α by T and B cells were assessed by intracellular staining and flow cytometry. The results showed that upon re-stimulation *in vitro* with PMA and ionomycin T cells isolated from mice maintained in CV facilities tended to express higher levels of CD69 and MHC I molecules compare to mice from SPF facilities. B cells obtained from CV facilities and re-stimulated *in vitro* with PMA and ionomycin tended to express higher levels of CD69, MHC I and MHC II molecules, but expressed significantly lower levels of CD86 molecule compared to cells isolated from mice that have been kept in the SPF facilities (Figure 3.17 A-E).

In addition, B cells isolated from mice maintained in SPF facilities were found to express significantly higher levels of IFN-γ and TNF-α compared to those isolated from CV facilities. On the other hand, T cells obtained from mice kept in the CV facilities expressed significantly higher level of IL-10 compared to those isolated from mice maintained in SPF facilities (Figure 3.18 A-D).

These results suggest that B cells from CV mice might be more activated than SPF cells, and that there is a Th1 bias in SPF mice compared to CV mice.

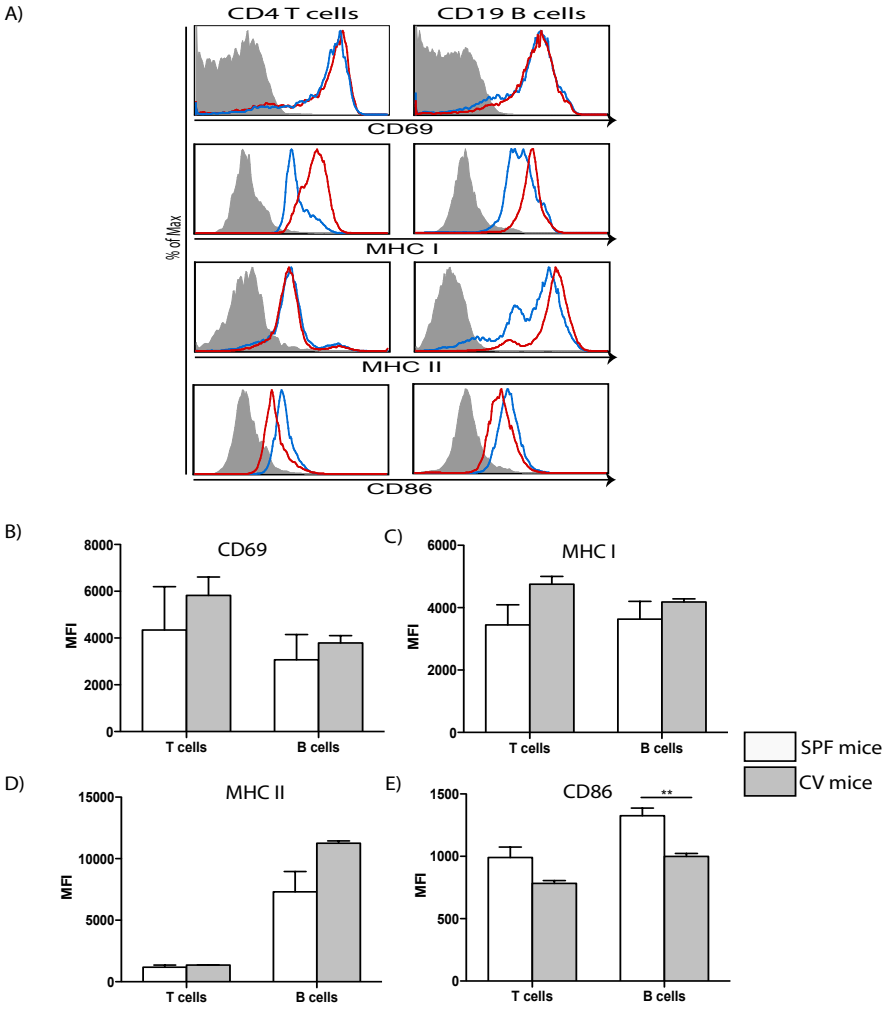

Figure 3.17 The expression of co-stimulatory molecules on B and T cells isolated from CV and SPF mice following re-stimulation *in vitro*. Spleens were collected from mice that had been kept in the SPF and the CV facilities. The splenocytes were activated for 5hrs with PMA & ionomycin then stained with anti-CD19, anti-CD4, anti-MHC I, anti-MHC II, anti-CD86 and anti-CD69 Abs. (A) Representative histograms of CD69, MHC I, MHC II, CD86 expressionon on $CD4^+$ T cells (left) and $CD19^+$ B cells (right), obtained from mice kept in the SPF (blue) and in the CV (red) facilities, isotype controls are shown as solid grey histograms. (B-E) Histograms displaying the MFI of expression of co-stimulatory molecules on $CD4^+$ T cells and $CD19^+$ B cells isolated from mice housed in SPF and CV facilities, (B) CD69, (C) MHC I, (D) MHC II, (E) CD86 expression. Graphs show mean± SEM, n=3. Statistics were calculated by t test, **P<0.005.

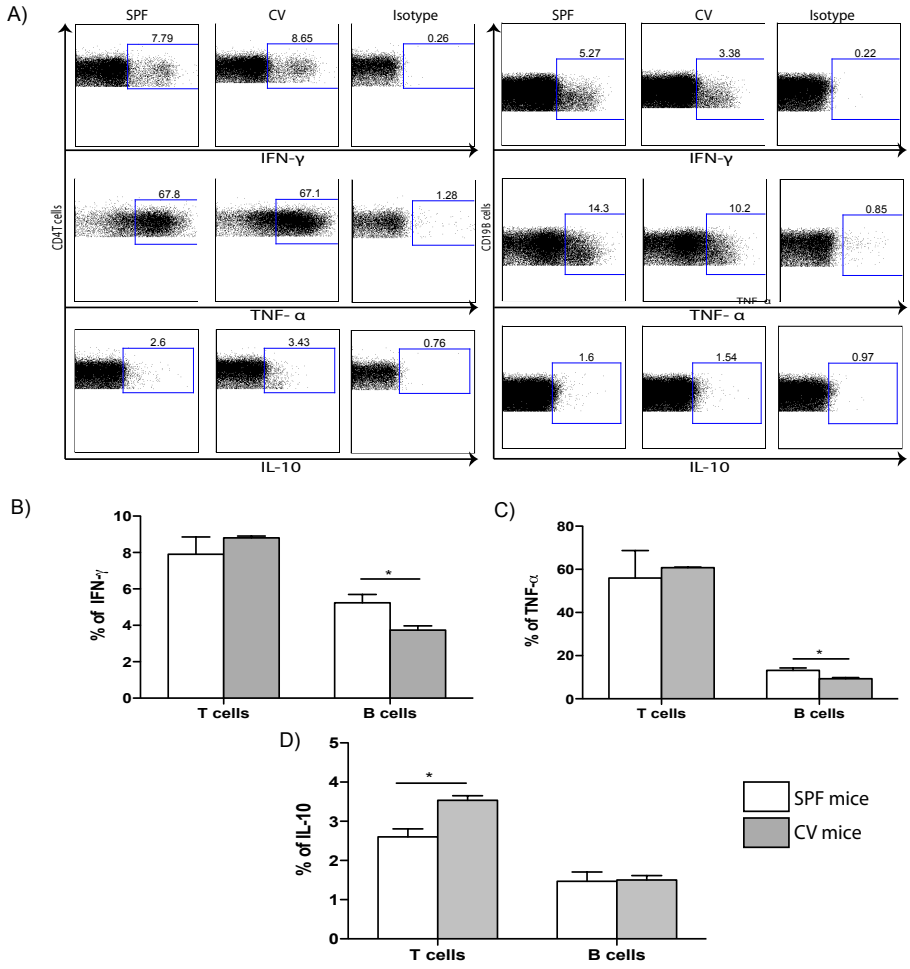

Figure 3.18 Differences in the cytokines response to *in vitro* activation of splenocytes isolated from mice kept in SPF and CV facilities. Spleens were collected from mice that had been kept on the SPF and the CV facilities. The splenocytes were activated for 5hrs with PMA & ionomycin then stained with anti-CD19, anti-CD4 (extracellular), anti-IFN-γ, anti-TNF-α, anti-IL10 Abs (intracellular). (A) Representative plots of IFN-γ, TNF-α and IL-10 expression by $CD4^+$ T cells and $CD19^+$ B cells isolated from mice maintained in SPF and CV facilities. (B-D) Histograms shows mean± SEM percentage of expression of, (B) IFN-γ, (C) TNF-α (D), IL-10 by $CD4^+$ T cells and $CD19^+$ B cells isolated from mice housed in SPF and CV facilities. n=3. Statistics were calculated by t test, *P<0.05.

3.9 Mice kept in CV facilities have different balance of microbiota and higher percentage of GC B cells than mice housed in SPF facilities.

Previous reports have shown that commensal microbiota, in particular some gut microbiota, influence the development of immune responses and protect from diseases [278,279]. However, how and whether commensal microbiota contribute in the development of such atopic diseases is not clear. Also, it has been reported that the differences in gut microbiota has affected stimulated and non-stimulated B cells isolated from the spleens and peyer's patches (PPs) of mice kept in CV facilities to secrete higher levels of Ig compared to mice kept in germ free facilities, suggesting that microbiota has an impact on B cells function [280]. Therefore, I sought to determine if the remarked variations I found in the make up of the immune system and cytokines profile between mice housed in the SPF and CV facilities are associated with variation in the make up of their gut microbiota, and if these changes have any influence on the level of B cell activation (GC B cells). Here, mice were kept in SPF and CV facilities for 8 weeks, subsequently, they were sacrificed and fecal samples, spleens, lymph nodes and PPs were collected. Fecal samples were used to identify the gut microbiota using FISH (see M&M), while the spleens, lymph nodes and PPs were used to determine the percentage of GC B cells. Figure 3.19 A shows that mice housed in CV facilities expressed significantly different levels of gut microbiota, and their spleens and PPs also expressed higher levels of GC B cells (Figure 3.19 B), compared to mice kept in SPF facilities.

Collectively, housing the mice under different level of sterility has resulted in the observed differences in the gut microbiota and the levels of B cells activation,

suggesting that microbiota has an impact on B cells activation and therefore function.

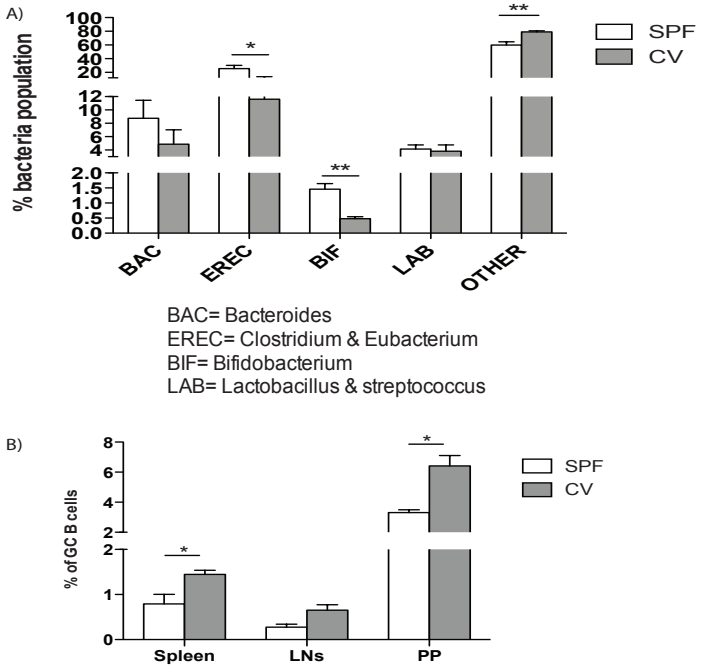

Figure 3.19 Mice kept in CV have different balance of microbiota and different level of GC B cells than mice housed in SPF facilities. Spleens, LNs, PPs & fecal samples were collected from B6 mice kept in SPF & CV facilities. (A) Histogram showing the gut microbiota in mice kept in SPF & CV facilities. (B) Histogram showing the percentages of GC B cells in spleens, LNs & PPs isolated from mice housed in SPF & CV facilities. Graphs show mean± SEM, n=3. Statistics were calculated by t test, *P<0.05 & **P<0.005.

Discussion

Up to date, the regulatory role of B cells in transplantation has not been extensively investigated. Indeed, B cells have been primarily thought of as the source of alloantibodies that activate complement and varieties of effector cells [281]. The presence of antibodies may simply reflect B cell activation, however, B cell effector mechanisms in the allo-response may be antibody independent. B cells do not influence the immune response solely through the production of antibodies, but also via cytokine production or by presenting antigens [125].

In this chapter, I focused on the regulatory role of B cells in transplantation. Furthermore, I have provided insights into the mechanisms by which B cells could mediate regulation in transplantation.

In a CIA model, Evans and colleagues compared the percentages of IFN-γ expressing $CD4^+$ T cells when cultured alone and when co-cultured with B cell subsets. They found that among all B cell subsets, T2-MZP B cells were the only subset capable of down regulating IFN-γ expressing $CD4^+$ T cells. Furthermore, they studied the correlation between IL-10 and T2-MZP B cell suppressive function, and found that T2-MZP B cell function in a contact-independent and IL-10-dependent manner [146]. Moreover, it has been documented that T2-MZP B cells were also shown to be regulatory in the MRL/lpr mouse model, and also that the equivalent population in humans has been associated with tolerance [149].

Therefore I sought to determine whether T2-MZP B cells could provide the same protective role in transplantation as they do in autoimmune models, and whether this role is IL-10 dependent as reported previously. I have shown in this

chapter that B cell subsets isolated from B6 mice maintained in the SPF facility did not suppress TNF-α expressing $CD4^+$ T cells obtained from B6 mice living in the same facility and stimulated *in vitro* by CD3/CD28 beads, plate bounded anti-CD3 antibody, or allo-DCs (Figure 3.3). The lack of regulation by B cells was independent from the capacity of the different B cell subsets to produce IL-10 (Figure 3.2). Since activating the T2-MZP B cell subset in lupus model with anti-CD40 mAbs resulted in an enhanced suppressive effect compared to the other B cell subsets [146149], I tested whether T2-MZP activated B cell subset stimulated with LPS and CPG can regulate T cell responses. Although activated T2-MZP B cells expressed the higher level of IL-10 compared to non-activated T2-MZP B cells (Figure 3.4 A), no regulatory function was exerted by this B cell subset or any other B cell subsets (Figure 3.4 B). Moreover, although MZ B cells expressed highest levels of IL-10 they still did not show any capacity to regulate TNF-α production by T cells (Figure 3.4 B & C). These data suggest that under these conditions there is no regulatory effect exerted by CPG/LPS stimulated $IL\text{-}10^+$ B cells on activated TNF-α expressing $CD4^+$ T cells. Additionally, the adoptive transfer of any of the B cell subset isolated from B6 mice housed in the SPF facility, into B6 recipients of B6-K^d skin grafts maintained in the same facility, failed to prolong skin graft survival (Figure 3.13). According to the hygiene hypothesis, which is also known as microbial exposure hypothesis, the improved hygiene and sanitary levels in developed countries lead to the rise in allergies and autoimmune diseases [282]. This hypothesis focuses on the role of microorganisms in tolerising the immune system to non-pathogenic and self-antigens [282]. Accordingly, Shimomura *et*

al. studied the influence of housing mice in CV and SPF facilities on the regulatory function exerted by B-1 B cells in chronic colitis. They found that maintaining mice in CV facilities suppressed colitis compared to mice maintained in SPF facilities, and that this protection from colitis was dependent on the presence of B cells as B cell deficient mice continued to develop colitis even in the CV facilities [274].

In line with Shimomura *et al*. I found that T2-MZP and T1 B cells obtained from mice kept in the CV facility were able not only to suppress TNF-α expression by CD3/CD28 bead and allo-DCs activated $CD4^+$ T cells *in vitro* (Figure 3.5), but also to significantly prolong B6-K^d skin graft survival on B6 recipients maintained in CV facility (Figure 3.14 B). These results suggest the possibility that cells separated from mice in CV facility have been primed and educated *in vivo* to exert the immune regulatory functions required. However, how T2-MZP and T1 B cell interactions with microorganisms have driven these cells to become regulatory is still to be investigated.

One possibility to explain the effect that housing mice in CV facilities has on the function of regulatory B cells is that microorganisms can activate B cells directly via their TLRs. Signals provided by microbes might be essential for the development and effector functions of T2-MZP and T1 B cells. It has been reported that TLR ligation on B cells can directly stimulate and mediate B cells to produce cytokines and antibodies [283]. Apoptotic cells (ACs) express TLR-9 ligands and can down-regulation inflammation caused by the innate immune cells [284,285,286,287,288] and protect mice from inflammation induced during autoimmune diseases [289]. A recent report has shown that stimulating B cells

with ACs induces B cells to produce IL-10 [264]. Additionally, the report documented that B cells isolated from mice whose B cells lack the expression of TLR-9 failed to produce IL-10 upon ACs stimulation [264]. This study further supports the important role that TLRs play in B cell responses. However, to confirm this possibility further work is required. For instance, keeping mice expressing MyD88$^{-/-}$ B cells in CV facility, and examine whether MyD88$^{-/-}$B cells in particular T1 and T2-MZP can provide the same suppressive role *in vitro* and *in vivo* as WT T1 and T2-MZP B cells.

The results in this chapter have also shown that T2-MZP and T1 B cells isolated from mice kept in CV, SPF facilities, or Gal-1$^{-/-}$ mice maintained in CV facility expressed similar levels of IL-10, but only B cells isolated from those mice housed in CV facility showed regulatory effect *in vitro* (inhibiting TNF-α expressing CD4^{+} T cells) and *in vivo* (prolonging allograft survival). Moreover, although MZ B cells expressed the highest level of IL-10 compared to other subsets obtained from mice kept in SPF, CV, or Gal-1$^{-/-}$ housed in CV facilities, they exhibited little to no regulatory function *in vivo* or *in vitro*. Additionally, pre-activating B cell subsets *in vitro* to express higher levels of IL-10 failed to drive T1 or T2-MZP B cell regulatory function (Figure 3.4). These results suggest that IL-10 might not be the major mechanism involved in T2-MZP and T1 B cells regulatory function. This is in contrast with what has been documented in autoimmunity where it has been found that T2-MZP B cell function in a contact-independent and IL-10 dependent manner [146]. My results also disagree with a recent report in the field of transplantation, where anti-Tim-1 treated JHD mice (B cell deficient mice) showed earlier allograft rejection, however, re-constituting

the mice with WT B cells, but not IL-10$^{-/-}$ B cells, prolonged the allograft survival, indicating that IL-10 plays a key role in the regulatory function of B cells in that model [173]. However, it has also been reported that inducing tolerance to cardiac transplant in mice using anti-CD45RB monotherapy is B cell dependent and that IL-10 production by B cells was in fact counter-regulatory and IL-10 neutralization improved graft outcome [318]. One can only postulate that B cells provide their regulatory function through different mechanisms according to the model and how B cells have been primed. To confirm the IL-10 hypothesis, IL-10$^{-/-}$ mice might be helpful to verify that IL-10 is not the major player in the model presented in this thesis. However, housing IL-10$^{-/-}$ mice in CV facilities would cause severe inflammation in the mice making it difficult to use them for these experiments.

Studies have shown that Gal-1 plays a key role in B cell function, in terms of immunoglobulin production [205], development [206], and their influence on T cells [207]. In addition Gal-1 has been discovered as a molecule involved in Treg suppressive function [196]. Therefore, I investigated whether Gal-1 was involved in T2-MZP and T1 B cell regulatory function. First, I confirmed by using western blot that B cells isolated from spleens of B6 mice expressed Gal-1 (Figure 3.8). This result is in agreement with the published work of Zuniga *et al.* in which demonstrated that B cells express Gal-1 [207]. Since this experiment required at least 0.5-1 million of cells after collection and washing, my attempt to examine Gal-1 expression in B cell subsets failed due to the low numbers of cells obtained after purifying the B cell subsets.

Next, I demonstrated that Gal-1$^{-/-}$ T2-MZP and T1 B cells lack suppressive

capacity (Figure 3.9). Moreover, the adoptive transfer of wild type T2-MZP B cells prolonged MHC I mismatched skin grafts significantly compared to PBS controls or mice that received Gal-1$^{-/-}$ T2-MZP B cells. Mice that received Gal-1$^{-/-}$ T2-MZP B cells rejected their graft even earlier than the controls (Figure 3.14 C). The results were similar when the effect of Gal-1 on the capacity of T1 B cells to prolong graft survival were investigated. However, the difference in skin survival between mice that received WT T1 B cells and Gal-1$^{-/-}$ T1 B cells was not significant, and a few additional experiments may be required to reach a significant difference (Figure 3.14 C). These results suggest that the expression of Gal-1 is essential for T2-MZP, and probably T1, B cell regulatory function. Thus, the role of Gal-1 as a functional regulatory molecule for Tregs was extended in this study to B cells [196]. However, the mechanism behind the regulatory role of Gal-1 during transplantation remains to be investigated.

As previously reported Gal-1 plays an important role in B cell development by anchoring the interaction between stromal cells integrins and pre-BCR on B cells providing survival signals during their development [208]. This might indicate that the observed functional defect in T2-MZP and T1 B cells in the transplant model presented here may be due to the altered interaction times or interaction kinetics between BM stromal cells and pre-B cells during B cell development in Gal-1$^{-/-}$ mice that might resulted in the generation of dysfunctional B cells. This might also explain the significant increase in the percentage of T2-MZP B cells in Gal-1$^{-/-}$ mice compared to WT mice (Figure 4.1). The loss of regulatory capacity by Gal-1$^{-/-}$ B cells might also be due to the requirement for Gal-1 in B cell intracellular signaling pathways (this point is

addressed in the next chapter in more detail). Exogenous Gal-1 has previously been reported to have an inhibitory effect on NF-κB activation in T cells, in particular Th1 T cells [266]. In addition, It has been documented that Gal-1 can interact with Ras at the cell membrane and activate the ERK pathway [187]. Another possible mechanism is that Gal-1 production by T2-MZP B cells is essential in shifting T cell responses from Th1 and Th17 to Th2. Toscano *et al*. have shown that Th1 and Th17, but not Th2, express on their surface a set of glycans that trigger cell death signal upon binding to Gal-1 [203]. Also, it has been reported that Gal-1 secreted by Th2 cells induced selective apoptosis of Th1, whereas Gal-1 produced by Th1 mediate secretion of Th2 cytokines [202]. Thus, the lack of regulatory function displayed by Gal-$1^{-/-}$ T2-MPZ B cells may be a result of their inability to secrete Gal-1 to directly inhibit T cells.

I attempted to block extracellular Gal-1 during the co-culture of allo-DCs, $CD4^+$ T cells and B cell subsets by using lactose. However, adding lactose to the control culture containing allo-DCs and TNF-α expressing $CD4^+$ T cells lead to a decrease in the percentage of TNF-α expressing $CD4^+$ T cells compared to a culture under the same conditions but without lactose. This result suggests that the presence of lactose in culture interfered with the interaction of T cells and allo-DCs.

Several co-stimulatory molecules on the surface of B cells have been identified to be up-regulated upon B cell activation, such as the B7 family molecules (CD80/CD86), CD40, ICAM-1, LFA-1 and VCAM-1 [291,281]. CD80 and CD86 molecules are of the best defined among these molecules, and it is well documented that their engagement with CD28 on T cells plays an important role

in T cell proliferation, cytokine production and their development to effector cells. On the other hand, it has been recently reported that CD80 and CD86 are essential for inhibiting and enhancing B cell activities, respectively [291]. In the present study, I have investigated the possible role of B7 molecules in B cell subsets regulatory function. Here, CD3/CD28 bead activated TNF-α expressing $CD4^+$ T cells were co-cultured with WT B cell subsets with or without anti-CD80 and anti-CD86 neutralizing antibodies. The results showed that by neutralizing B7 molecules T2-MZP B cells significantly loss their suppressive capability compared to controls (Figure 3.10 A). These results point to the important role of B7 molecules in T2-MZP B cells regulatory function. This experiment was in the absence of any type of APC because the neutralizing anti-B7 mAb inhibited allo-DCs activated TNF-α expressing $CD4^+$T cells.

Another mechanism that I have investigated in this chapter is the involvement of alloantibody to graft rejection. Alloantibodies have been known for their central role in provoking transplant rejection through complement-dependent and complement-independent pathways [63]. Here, allo-IgGs were measured in sera collected (one month after transplant) from mice kept either in SPF or CV facilities either received no B cells or received adoptive transfers of B cell subsets. Figure 3.13 & 3.14 showed no correlation between allo-IgG and graft rejection, and both groups of mice (SPF vs. CV) produced allo-IgG. These results suggest that the presence of allo-IgG does not correlate with T2-MZP B cells ability to prolong skin survival.

In this chapter, I have also found that MZ B cells expressed the highest level of IL-10 and were capable of suppressing TNF-α expressing $CD4^+$ T only when T

cells were activated with beads, but not with allo-DCs. Furthermore, their adoptive transfer significantly prolonged MHC I mismatched skin graft survival. However, transfer of T2-MZP B cells provided a significantly better survival rate than MZ B cells, and the prolongation provided by MZ B cells, although significant, was not very different from the controls.

T1 B cells were also able to suppress and there are a number of possible reasons to explain these findings. First, T1 B cells are very immature cells, and it is possible that once injected or stimulated they mature into T2-MZP B cells. However, this hypothesis needs to be investigated further. Secondly, it could be related to their survival capacity. T1 B cells do not survive very well through the process of isolation and FACS sorting, so it maybe that the cells injected are in fact apoptotic, and, as described above, apoptotic cells can possess an immune-regulatory function. Further investigation could clarify this point, such as labeling the cells with CFSE to monitor their survival, or by phenotyping the cells again after *in vivo* injection or culture.

The fact that T2-MZP and T1 B cells function differently simply as a result of housing the mice in the two facilities where the major difference is the level of sterility, lead me to investigate their immunological make up, their responses to stimuli *ex vivo* and their gut microbiota colonization.

As previously reported gut microbiota can shape the immune system by strengthening the cooperation between adaptive and innate immune systems, so they establish strong gut barrier and prevent the microbiota from invading the host [292]. Also, it has been documented that alteration in the gut microbiota during childhood modulates the development of the immune system

[293,294]. Moreover, immune cells continually recognize microbial antigens via their TLRs, which initiate complex interactions between microbiota and the immune cells [295]. These interactions have been reported to shape immunity throughout life [296,297]. Therefore, I next sought to determine if housing the mice under different hygienic conditions (CV vs. SPF) could effect the gut microbiota composition. Here, I found that mice housed in CV facility had a different composition of gut microbiota than mice kept in SPF facilities (Figure 3.19 A). These results were associated with a significant increase in lymph node memory $CD4^+$ and $CD8^+$ T cells in mice kept in CV facility compared to SPF facility (Figure 3.15 E), indicating more productive immune responses in mice kept in CV facility. Furthermore, re-stimulating splenocytes isolated from mice housed in SPF facility showed that B cells expressed significantly higher levels of TNF-α and IFN-γ compared to B cells isolated from CV housed mice, and significantly higher level of IL-10 expressing $CD4^+$ T cells in CV housed mice compared to mice kept in SPF facility (Figure 3.18). These data suggest that B cells from mice housed in CV facility tended to shift the Th1/Th2 balance toward Th2 compared to B cells from SPF facility. Also, I found that the percentages of GC B cells are significantly higher in the PPs and spleens of mice maintained in CV facility compared to mice kept in SPF facility (Figure 3.19 B), further suggesting the higher state of activation of B cells in the gut when derived from mice kept in the CV compared to the SPF facility. Moreover, B cells in the lymph nodes isolated from mice kept in CV facility expressed significantly higher levels of MHC I and MHC II molecules compared to SPF facility (Figure 3.16 D). However, the housing conditions did not influence B cell

development (Figure 3.15 A). These results are in line with a recent study investigating the influence of microbial colonization on B cell development and function that investigated mice kept in germ free and CV facilities [280]. The authors reported that the differences in gut microbiota had no effect on B cell development. B cells isolated from the spleens and PPs of mice kept in CV facilities secreted higher levels of Ig however, suggesting that microbiota has an impact on B cells function [280].

Collectively, the results in this chapter showed that the level of sterility in which the mice are kept influences gut microbial homeostasis resulting in more productive immune responses. Furthermore, B cells from mice housed in CV facility acquired regulatory function allowing them to suppress TNF-α expressing $CD4^+$ T cells *in vitro* and to prolong allograft skin survival *in vivo*. Whether B cells can "acquire" regulatory function in other ways such as during transplantation tolerance induction, as suggested from work using blood from transplanted renal patients that become tolerant to the graft [56], will be addressed in Chapter 5. Finally, Gal-1 expression was necessary for the regulatory function of B cells.

Chapter 4- Results 2

Chapter 4

In the previous chapter I have shown that B cell subsets, apart from FO B cells, can have regulatory function and prolong transplant survival but only when isolated from mice housed under non-hygienic conditions. These findings were associated with a difference in the hemostasis between gut microbiota of mice kept in SPF and CV facilities. This imbalance in gut microbiota suggested a possible role for TLR signaling that might educated the B cells *in vivo* to exhibit their regulatory function. Signaling via TLR molecules has been shown to play an important role in modifying the responses of B cells, such as their cytokine secretion, antibody production and their capacity to present antigen [251]. Cytokine secretion by B cells upon TLRs ligation can influence Th cells polymerization at early stage. In *Salmonella enterica* model, B cell activation through TLRs without antigen requirement were essential for Th1 generation, while antigen presentation and BCR were important for Th1 memory formation. Infecting MyD88$^{-/-}$ mice with *S.enterica*, and re-stimulating purified B cells from this mice with heat-killed bacteria showed negligible secretion of IL-6, IL-10 and IFN-γ, suggesting complete dependency on TLRs signaling in B cells to secrete those cytokines [263]. Moreover, Miles *et al,* found that apoptotic cells (AC), which express chromatin complex and stimulate TLR-9, when treated with DNase and adoptively transferred did not protect the mice from arthritis compared to the controls that received non-treated AC. Moreover, in TLR-9$^{-/-}$ mice AC-administration did not mediate protection from EAE [264]. Re-stimulation of splenocytes isolated from TLR-9$^{-/-}$ mice with EAE at day 12 showed a significant drop in IL-10 and higher levels of IL-17 and IL-6 compared

to WT mice. Moreover, protection from EAE by AC was restored by the adoptive transfer of WT B cells together with AC, but not when B cells were coming from TLR-9$^{-/-}$ mice, suggesting that TLR-9 is essential for B cells to maintain self-tolerance in heath [264].

The engagement of TLRs triggers various signaling pathways leading to the up-regulation of co-stimulatory molecules and cytokine production [217,229]. Co-stimulatory molecules shape B cell responses and their functions in the immune system. For example, antigen specific B cells can be very potent antigen presenting cells by presenting antigens in a specific manner via their MCH I and II to both CD4 and CD8 T cells [126,127,128]. Therefore, B cells have the ability to shape the level of T cell responses [260,275,126,127]. Cytokine production by B cells contributes as well to the development of the lymphoid microenvironments [298], and in shaping T cell responses [275,276,277]. For example, IL-10 secretion by B cells can down regulate inflammation associated with autoimmune diseases via modulating T cell function [145,146,149,169].

Finally, I have shown in Chapter 3 that the expression of Gal-1 is essential for B cell regulatory function and that B cells that lack Gal-1 expression from mice in CV conditions cannot inhibit the immune responses both *in vitro* and *in vivo*. Gal-1 has already been reported to play a key role in B cells development [208]; Abs production [205] and Gal-1 producing B cells can regulate T cell responses [207].

Bringing all these results together, I addressed in this chapter the consequence of TLR-4 and TLR-9 ligation on B cell functions with particular attention to co-

stimulatory molecules expression and the production of the immunoregulatory cytokine IL-10.

Note: all the cells and tissues in this chapter have been obtained from the SPF facilities only.

4.1 B cell proportions in Gal-1$^{-/-}$ mice.

As I have previously shown in chapter 3 that B cells express Gal-1. In this chapter I investigated the consequence for B cell stimulated by TLRs of lack of Gal-1-expression. However, first I evaluated the proportions of B cells and their development in WT and Gal-1$^{-/-}$ mice. I isolated spleens, lymph nodes and PPs from Gal-1$^{-/-}$ mice and WT mice and compared the percentages of T2-MZP, T1, MZ, FO and GC B by flow cytometry. I found significantly higher percentage of T2-MZP B cells, as a proportion of total B cells, in the spleens of Gal-1$^{-/-}$ compared to WT mice, whereas there were a lower percentage of GC B cells in the spleens of Gal-1$^{-/-}$ mice compared to WT mice (Figure 4.1 A, B and C). In contrast, WT and Gal-1$^{-/-}$ mice have the same percentages of B cells and B cell subsets in PPs and lymph nodes (Figure 4.1 D and E).

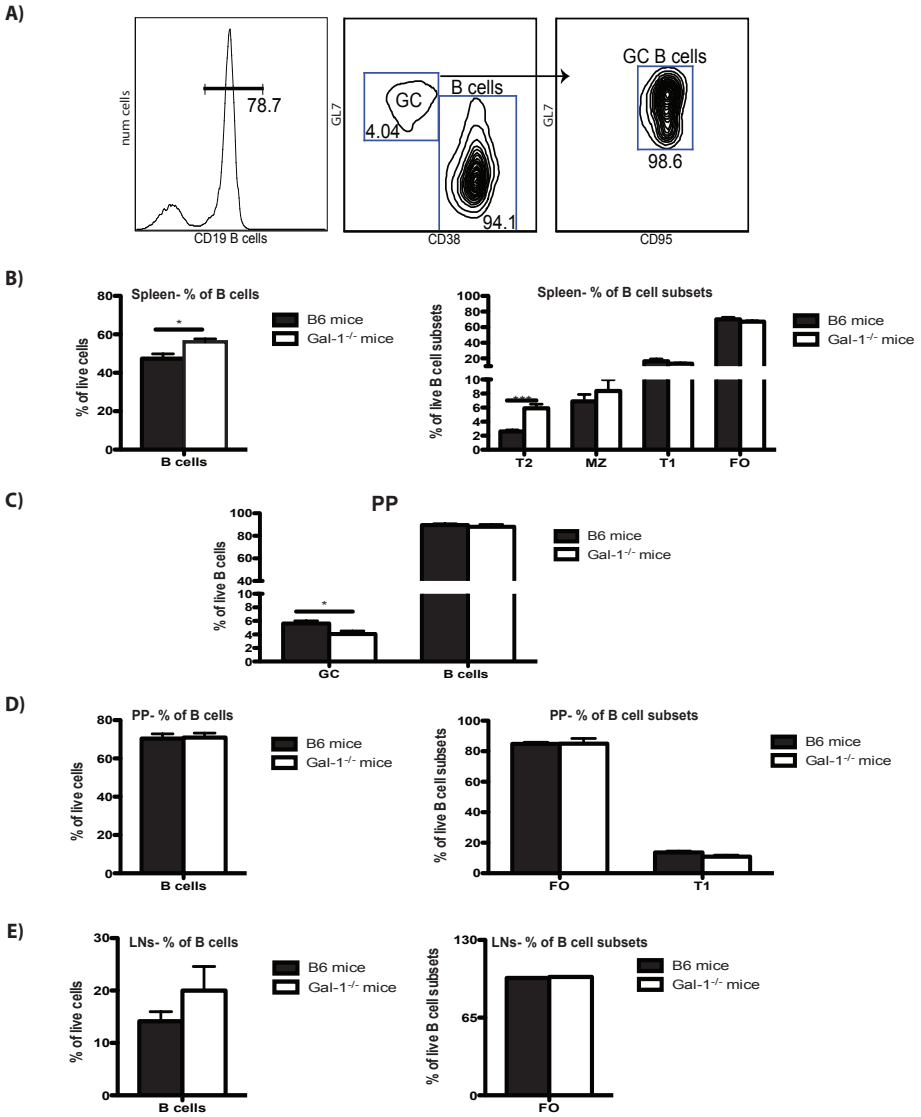

Figure 4.1 B cell proportions in Gal-1$^{-/-}$ mice. Spleens, LNs and PPs were isolated from naïve B6 and Gal-1$^{-/-}$ mice, and phenotyped using the following Abs: anti-CD19, anti-CD21, anti-CD24, anti-CD23, anti-CD38, anti-CD95 and anti-GL7 (GC B cells were CD19^{+}, GL7^{+}, CD38^{-} and CD95^{+}). (A) Representative FACS plots of GC gating strategies. Histograms displaying, (B) the percentages of total B cells and B cell subsets isolated from the spleens of B6 and Gal-1$^{-/-}$ mice (n=6), (C) the percentages of GC and B cells isolated from the PPs of B6 and Gal-1$^{-/-}$ mice (n=12), (D) the percentages of total B cells and B cell subsets isolated from the PPs of B6 and Gal-1$^{-/-}$ mice (n>3), (E) the percentages of total B cells and B cell subsets isolated from the LNs of B6 and Gal-1$^{-/-}$ mice (n=6). Statistics were calculated by t test, *P<0.05 & ***P<0.0005.

4.2 Gal-1$^{-/-}$ B cells express significantly higher levels of co-stimulatory molecules compared to WT B cells in response to LPS.

Co-stimulatory molecules are key in contributing to the immune response. It is known that antigen-specific B cells are very potent antigen presenting cells (APC) by concentrating antigens via their BCR and presenting them on their MHC class I and class II to $CD8^+$ and $CD4^+$ T cells, respectively [125,126,127,128]. B cells can provide additional signals for the activation and proliferation of T cells through CD80 and CD86 [135,136]. Therefore, I next examined whether Gal-1 expressed by B cell can contribute to co-stimulatory molecule expression following TLR stimulation. Here, I isolated B cells magnetically, and activated them with the following stimuli: LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. Subsequently, CD80, CD86, CD40 and MHC II expression were measured by flow cytometry. The results showed first of all that resting naïve Gal-1$^{-/-}$ B cells expressed similar levels of MHC and co-stimulatory molecules to WT B cells, with the exception of MHC I, which was expressed at lower levels on Gal-1$^{-/-}$ B cells (Figure 4.2). Following LPS stimulation however Gal-1$^{-/-}$ B cells expressed significantly higher levels of CD80, CD86, CD40 and MHC II compared to WT B cells. Gal-1$^{-/-}$ B cells expressed higher levels of CD86 and MHC II following stimulation with LPS. CD80 molecules were also expressed at higher levels when B cells from Gal-1$^{-/-}$ mice were stimulated with LPS and LPS plus anti-CD40 mAbs compared to WT B cells. Finally, CD40 molecules were also expressed at higher levels on B cells isolated from Gal-1$^{-/-}$ mice compared to

those isolated from WT mice following stimulation with anti-CD40, anti-CD40/LPS and anti-CD40/CPG (Figure 4.3).

These results suggest that Gal-1 might have a role in negatively regulating the expression of B cell co-stimulatory molecules in response to TLR-4 ligation.

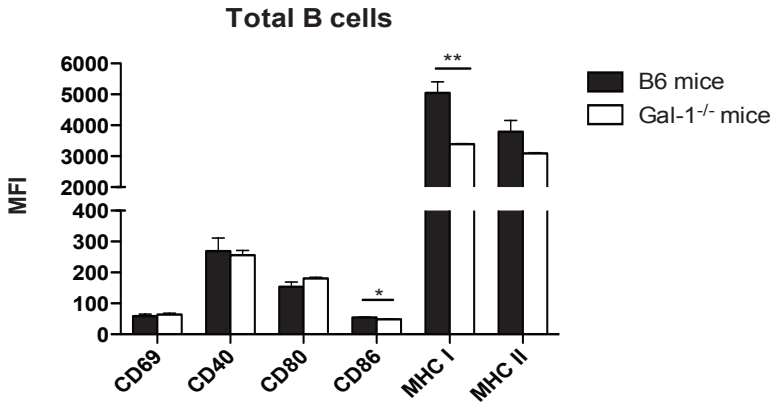

Figure 4.2 Co-stimulatory molecules expression on B cells isolated from spleens of naïve B6 and Gal-1$^{-/-}$ mice. Splenocytes were collected from naïve B6 and Gal-1$^{-/-}$ mice, and stained with anti-CD19, anti-MHC I, anti-MHC II, anti-CD80, anti-CD86, anti-CD40 and anti-CD69 Abs. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05 & **P<0.005.

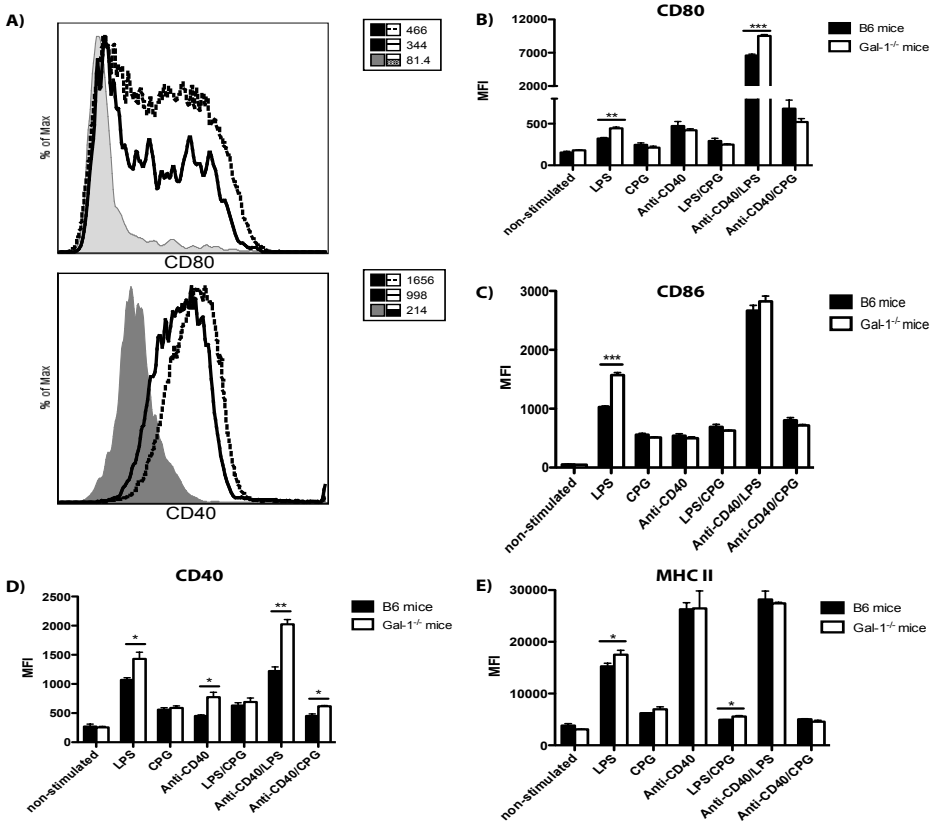

Figure 4.3 Gal-1$^{-/-}$ B cells express significantly higher levels of co-stimulatory molecules compared to WT B cells in response to LPS. B cells were isolated from spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. B cells were then stained with anti-CD19, anti-CD80, anti-CD86, anti-CD40 and anti-MHC II Abs. (A) Representative FACS histograms of the expression of CD80, CD40 on CD19 B cells isolated from B6 (soild line) and Gal-1$^{-/-}$ (dotted line) mice (isotype solid grey histograms). Histograms shows mean±SEM expression of, (B) CD80, (C) CD86, (D) CD40, (E) MHC II on non-stimulated and stimulated B cells from B6 and Gal-1$^{-/-}$ mice (n=3). Statistics were calculated by t test, *P<0.05, *P<0.005 & ***P<0.0005.

4.3 B cells isolated from the Peyer's Patches of Gal-1$^{-/-}$ mice express higher levels of CD80 and CD86 compared to WT B cells.

Given that Gal-1$^{-/-}$ B cells expressed higher levels of CD80 and CD86 than WT B cells following stimulation *in vitro* with LPS I investigated whether B cells recovered from sites of activation *in vivo* had the same phenotype of the B cells activated *in vitro*. In this experiment, I compared the expression of CD80 and CD86 molecules by WT and Gal-1$^{-/-}$ B cells isolated from PPs as these sites should contain B cells continuously activated by gut antigens and via their TLRs. In agreement with the *in vitro* results Gal-1$^{-/-}$ GC B cells expressed significantly higher levels of CD80 and a tendency to express higher levels of CD86 molecules (Figure 4.4)

To summarize Gal-1$^{-/-}$ B cells expressed higher levels of co-stimulatory molecules compared to WT B cells *in vitro* (LPS) and when obtained *ex vivo*. These results suggest that stimulation of B cells via TLR-4 ligand (LPS) in the absence of Gal-1 might results in a higher level of B cell activation.

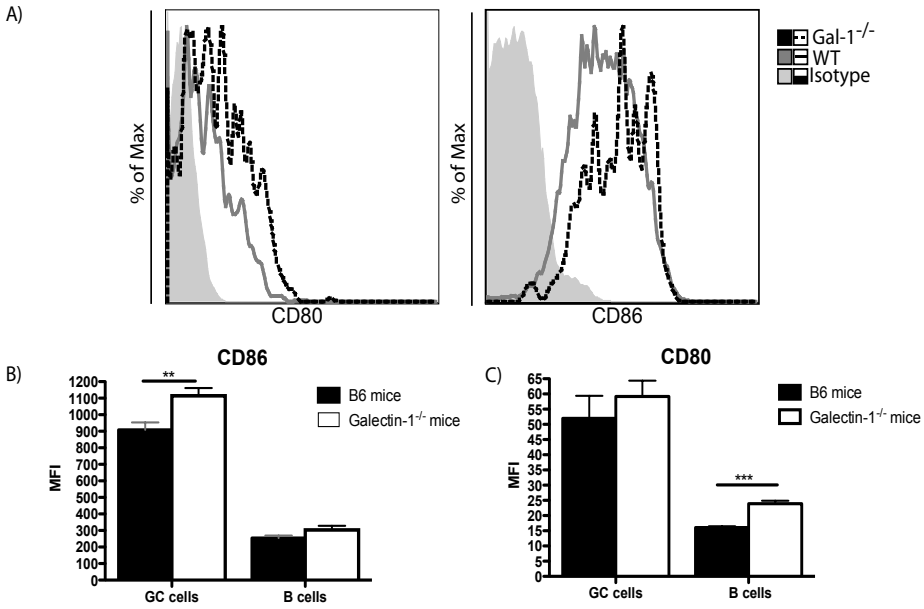

Figure 4.4 Gal-1$^{-/-}$ B cells express higher levels of CD80 and CD86 compared to WT B cells, *Ex vivo*. Cells were isolated from PPs of B6 & Gal-1$^{-/-}$ mice, and stained with anti-CD19, anti-CD38, anti-CD95, anti-GL7, anti-CD80 and anti-CD86 Abs. (A) Representative FACS histograms of CD80 and CD86 expression on CD19 B cells isolated from B6 (solid line) and Gal-1$^{-/-}$ (dotted line) mice (isotype is the solid grey histogram). Histograms displaying the expression of (B) CD86, & (C) CD80 on GC and B cells isolated from PPs of B6 and Gal-1$^{-/-}$ mice. Results displayed as mean± SEM, n=6. Statistics were calculated by t test, **P<0.005 & ***P<0.0005.

4.4 Different amount of IL-10 produced by Gal-1$^{-/-}$ B cells compared to WT B cells in response to CPG when two detection assays were used.

Cytokine production by B cells is an important mechanism in the development of lymphoid tissue microenvironments [298], and shaping T cell responses [275,276,277]. For example, IL-10 secretion by B cells can down regulate inflammation associated with autoimmune diseases via modulating T cell functions [145,146,149,169]. In addition, it has been reported that the adoptive transfer of a subset of B cells (T2-MZP) were capable of protecting from autoimmune diseases through IL-10 production [146,149]. Since I was interested in the suppressive role of B cells, which has been reported to be dependent on IL-10, I next determined if Gal-1 deficiency would have affected B cell capacity to produce IL-10. B cells were isolated magnetically from Gal-1$^{-/-}$ and B6 mice, and cultured with combinations of different stimuli, LPS, CpG and anti-CD40 for 48hrs. PMA and Ionomycin and brefeldin A were added for the final 4 hrs of culture after collecting supernatants for ELISA. The intracellular staining results in figures 4.5 A & B show that IL-10 expression was significantly lower in Gal-1$^{-/-}$ B cells at 48hrs compared to WT B cells in particular following stimulation with TLR-9 ligand CPG. However, the ELISA results in figure 4.5 C show that after ~48hrs Gal-1$^{-/-}$ B cells had tendency to secrete higher levels of IL-10 compared to WT B cells with most of the stimuli. The ELISA results were confirmed by using cytokine bead array. The cytokine bead array results demonstrated that Gal-1$^{-/-}$ B cells secreted significantly higher levels of IL-10 compared to WT B cells after ~48hrs of stimulation with CPG and LPS (Figure 4.5 D).

Given that intracellular staining provides a snapshot of what the cells are expressing at certain time points, while ELISA detects the cumulative cytokines produced over the culture period, the different results obtained with the two methods suggest that there is a difference in the kinetic of IL-10 production by Gal-1$^{-/-}$ and WT B cells.

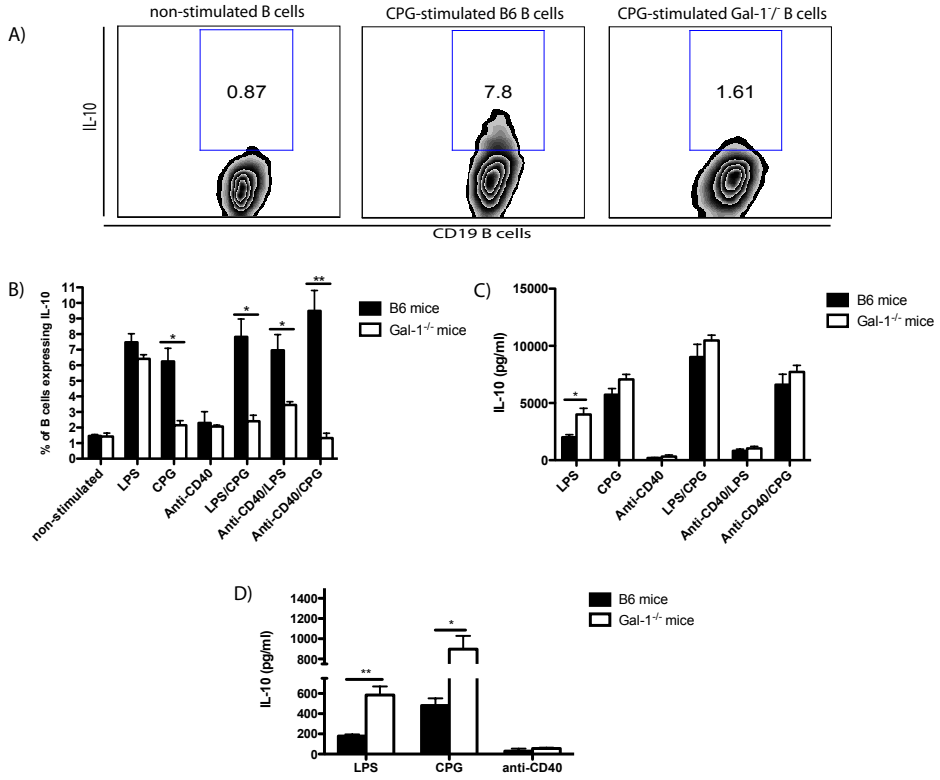

Figure 4.5 Gal-1$^{-/-}$ B cells have altered cytokine production after 48hrs re-stimulation *in vitro* compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture. supernatants were collected for ELISA before the addation of PMA and Ionomycin and brefeldin A. (A) Representative FACS plots of IL-10 expression by CPG activated and non-activated B cells isolated from B6 and Gal-1$^{-/-}$ mice. Histograms displaying, (B) percentage of B cells expressing IL-10, (C) IL-10 production by B cells as measured by ELISA, (D) IL-10 production by B cells as measured by cytomix. Results represent mean± SEM, n=3 except ELISA n=9. Statistics were calculated by t test, *P<0.05 & **P<0.005.

4.5 Gal-1$^{-/-}$ B cells exhibit different kinetics than WT B cells.

To investigate the hypothesis that Gal-1$^{-/-}$ B cells produced IL-10 with different kinetics to WT B cells, Gal-1$^{-/-}$ and WT B cells were cultured with combinations of LPS, CpG and anti-CD40 for 6, 24 and 48hrs. PMA and Ionomycin and brefeldin A were added for the final 4hrs of culture after collecting supernatants for ELISA. Intracellular staining showed a significant increase in IL-10 expression by Gal-1$^{-/-}$ B cells at 6hrs (Figure 4.6 A & B), and a trend of higher expression at 24hrs (Figure 4.6 C). However, a significant drop in IL-10 expression by Gal-1$^{-/-}$ B cells was observed at 48hrs particularly with CPG, compared to WT B cells (Figure 4.6 D). These results were also confirmed in ELISA, where Gal-1$^{-/-}$ B cells had a tendency of producing higher levels of IL-10 at 6 and 24hrs (Figure 4.7 A and B). Although I observed a higher level of IL-10 at 48hrs in the supernatants of Gal-1$^{-/-}$ B cells compared to WT B cells (Figure 4.7 C), the intracellular staining has demonstrated that Gal-1$^{-/-}$ B cells stopped expressing IL-10 at this time point. The differences in kinetics of IL-10 production between WT and Gal-1$^{-/-}$ B cells were not a result of differences in the viability of the B cells (Figure 4.8). FACS staining with live/dead fixable blue dead cell stain kit revealed no significant differences between Gal-1$^{-/-}$ and WT B cells at any time point or in the presence of any of the stimuli.

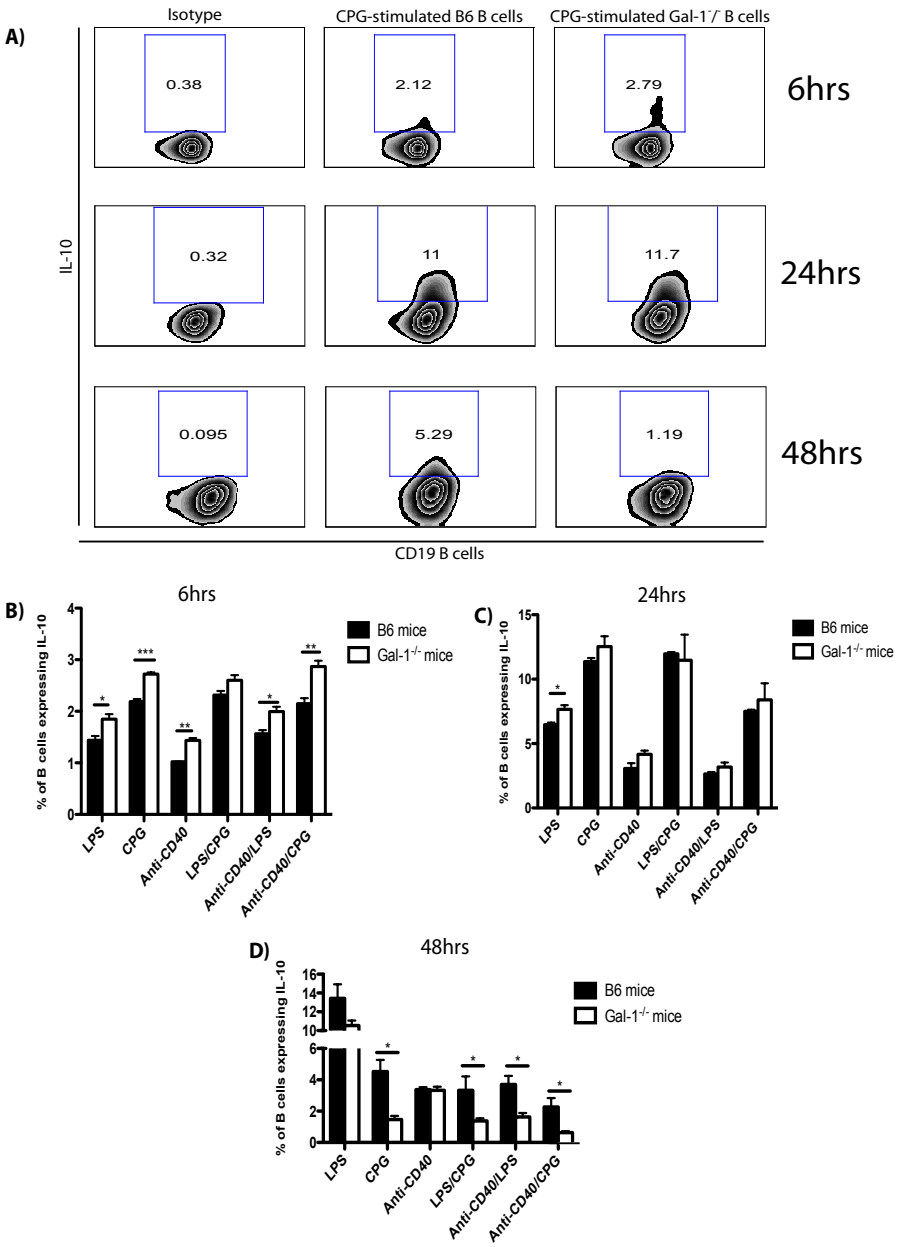

Figure 4.6 Gal-1$^{-/-}$ B cells exhibit different kinetics of IL-10 expression compared to WT B cells (ICC). B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 6hrs, 24hrs and 48hrs. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture, cells were then stained for CD19 and IL-10 (ICC). (A) Representative FACS plots of IL-10 expression by CPG activated B cells that were isolated from B6 and Gal-1$^{-/-}$mice for 6hrs, 24hrs and 48hrs. Histograms displaying the percentages of IL-10 expressing B cells after (B) 6hrs, (C) 24hrs and (D) 48hrs of incubation. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05, *P<0.005 & ***P<0.0005.

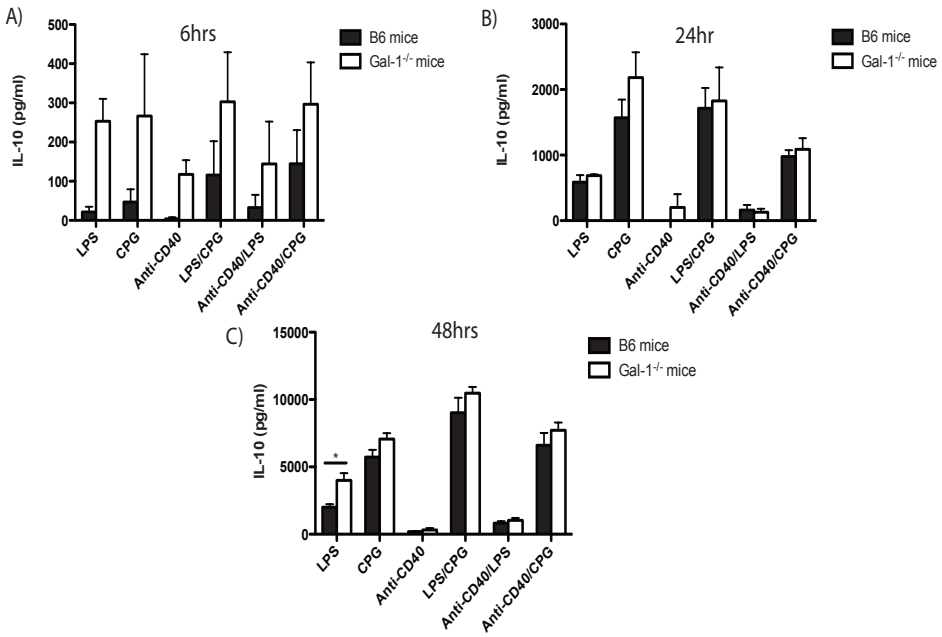

Figure 4.7 B cells without Gal-1 exhibit different kinetics of IL-10 production compared to WT B cells (ELISA). B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 6hrs, 24hrs and 48hrs. Subsequently, supernatants were collected for ELISA. Histograms displaying IL-10 production by B cells after (B) 6hrs , (C) 24hrs and (D) 48hrs of incubation. Results represent mean± SEM, n=3, except the 48hrs n=9. Statistics were calculated by t test, *P<0.05.

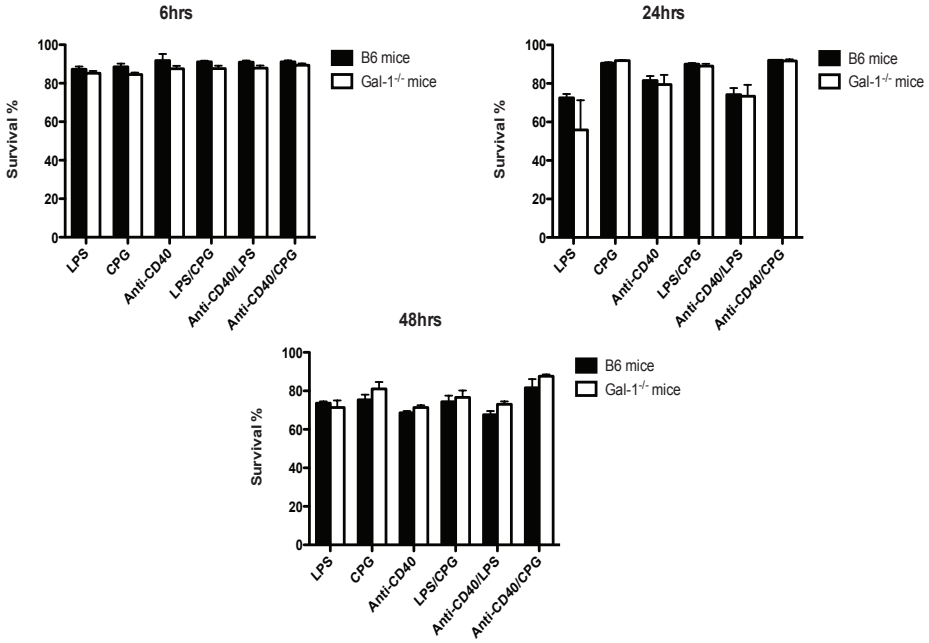

Figure 4.8 The survival of Gal-1$^{-/-}$ B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 6hrs, 24hrs and 48hrs. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture, cells were stained with CD19 and invitrogen LIVE/DEAD Fixable Blue Dead Cell Stain Kit to distinguish dead cells. Histograms displaying the percentages of live B cells after (A) 6hrs, (B) 24hrs and (C) 48hrs of incubation. Results represent mean± SEM, n=3. Statistics were calculated by t test.

4.6 Alterations in IL-10 expression by Gal-1$^{-/-}$ B cells compared to WT B cells following TLR-9 ligation appear to be localized particularly to T2-MZP & FO B cell subsets.

To identify which B cell subset is the most affected by Gal-1 deficiency, I looked at the expression of IL-10 by FACS purified B cell subsets following TLR stimulation. B cells were magnetically isolated from B6 and Gal-1$^{-/-}$ mice, and subsets were purified by flow cytometry. Cells were then cultured with combinations of LPS, CpG and anti-CD40 for 48hrs. PMA and Ionomycin and brefeldin A were added for the final 4hrs of culture after collecting supernatants for ELISA. Figures 4.9 A, B and C show that the same significant drop in IL-10 expression observed with total B cells was seen with T2-MZP and FO B cells, particularly with CPG stimulation. In figure 4.9 D is shown that IL-10 expression by T1 was also slightly decreased with most stimuli, however the differences were not significant and could be explained by the high degree of cell death by both WT and Gal-1$^{-/-}$ T1 B cells. There were no differences in IL-10 expression by MZ B cells isolated from Gal-1$^{-/-}$ or B6 mice (Figure 4.9 E).

The ELISA results in figure 4.10 show that over the course of 48hrs both WT and Gal-1$^{-/-}$ T2-MZP and FO B cells have secreted similar amounts of IL-10 even though Gal-1$^{-/-}$ T2-MZP and FO B cells have significantly reduced IL-10 expression (ICC) by this point.

These results suggest that Gal-1 deficiency affects T2-MZP and FO B cell IL-10 production more than T1 or MZ B cells.

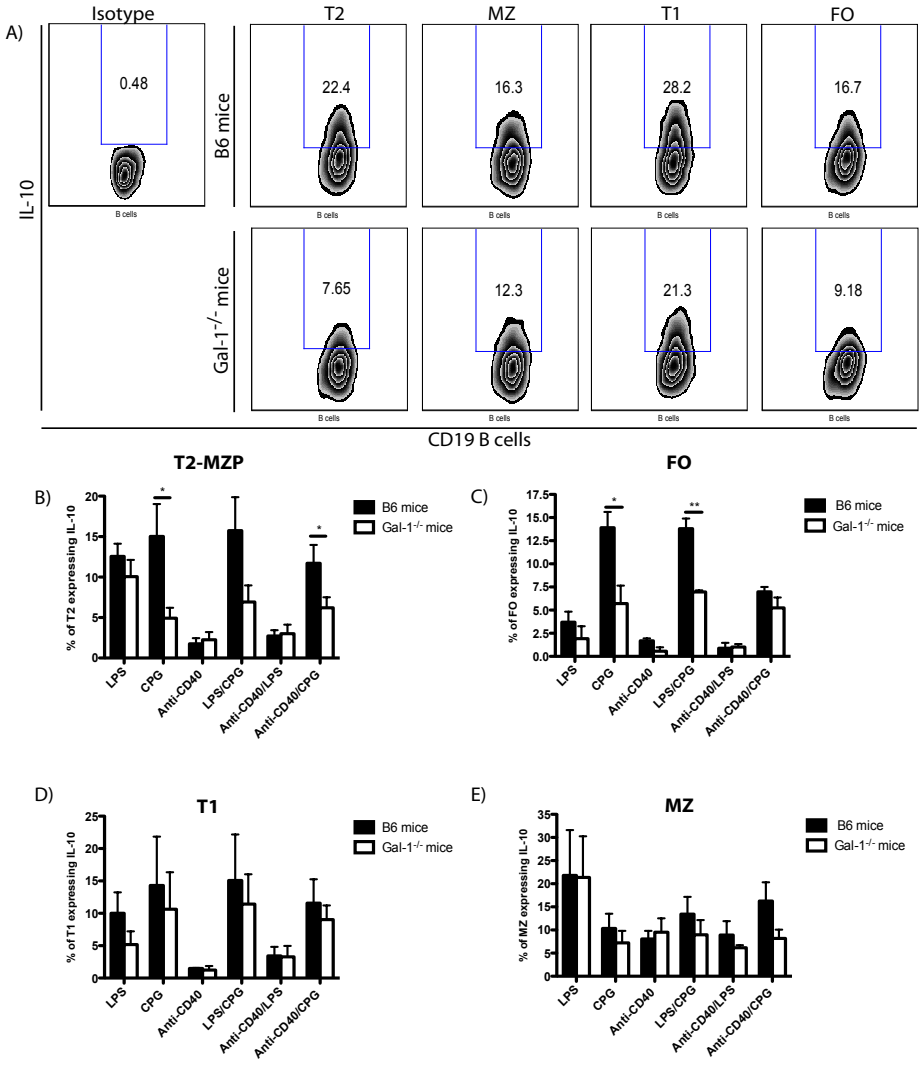

Figure 4.9 Gal-1$^{-/-}$ T2-MZP & FO B cell subsets exhibit a significant drop in IL-10 expression compared to WT B cells in response to CPG. B cells were isolated from spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting. B cell subsets were purified by FACS and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. PMA and Ionomycin and brefeldin A were added for the last 4 hours of culture, cells were then stained for CD19 and IL-10 (ICC). (A) Representative FACS plots of IL-10 expression by CPG activated B cell subsets isolated from B6 and Gal-1$^{-/-}$ mice. Histograms displaying the percentages of IL-10 expressing (B) T2-MZP, (C) FO, (D) T1, (E) MZ B cells after 48hrs. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05 & **P<0.005.

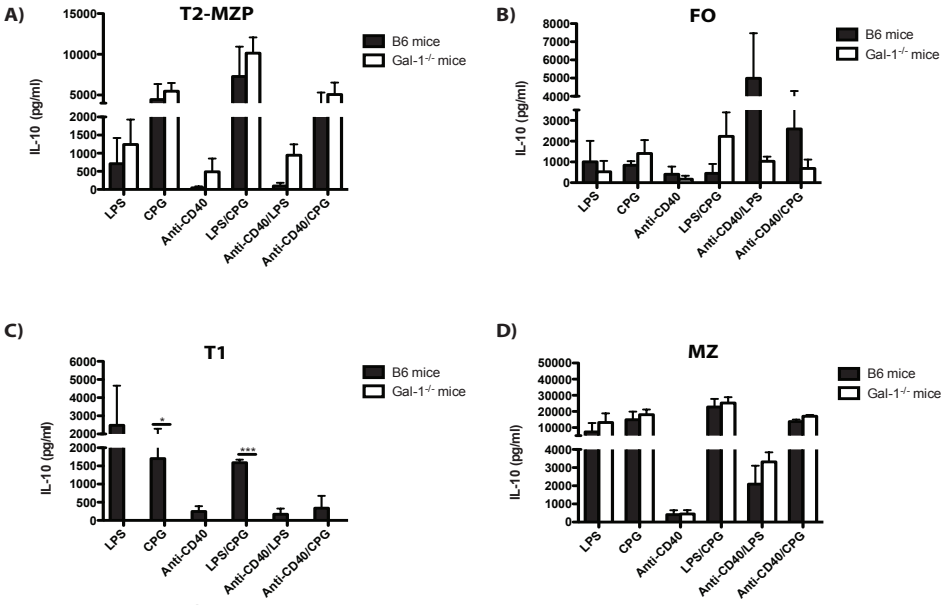

Figure 4.10 Gal-1$^{-/-}$ T2-MZP & FO B cell subsets exhibit a trend of higher IL-10 production compared to WT B cells in response to CPG (ELISA). B cells were isolated from spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting. B cell subsets were purified by FACS and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. Subsequently, supernatants were collected for ELISA. Histograms displaying IL-10 production by (A) T2-MZP, (B) FO, (C) T1 , (D) MZ B cells after 48hrs. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05 & ***P<0.0005.

4.7 Gal-1$^{-/-}$ B cells express the same level of TLRs and have the same capacity to uptake LPS as WT B cells.

To exclude the possibility that the functional differences between Gal-1$^{-/-}$ and WT B cells were simply due to reductions in the expression of TLR-4 and TLR-9 by Gal-1$^{-/-}$ B cells compared to WT B cells, I examined the expression of these two TLRs on Gal-1$^{-/-}$ and WT B cells. B cells were magnetically isolated, activated with combinations of LPS, CpG and anti-CD40 for 48hrs, and stained with anti-CD19, anti-TLR-4 and anti-TLR-9 antibodies (ICC). I found no differences in TLR-4 and TLR-9 expression between Gal-1$^{-/-}$ and WT B cells apart from a slightly reduced expression of TLR-4 following LPS stimulation in B cells from Gal-1$^{-/-}$ mice (Figure 4.11).

In view of the fact that Gal-1 is a carbohydrate-binding protein, and LPS is mainly lipid and carbohydrate [187,299], I tested whether the absence of extracellular Gal-1 influenced LPS uptake by Gal-1$^{-/-}$ B cells. B cells isolated from Gal-1$^{-/-}$ and B6 mice were incubated with LPS labeled with FITC and anti-CD19 Abs for 30 minutes, washed, and then FITC expression by CD19 B cells was evaluated by flow cytometry. The results showed no significant differences in LPS uptake between Gal-1$^{-/-}$ and WT B cells (Figure 4.12 B).

In summary, I did not observe marked differences in TLR expression, or in the level of LPS uptake, between Gal-1$^{-/-}$ and WT B cells, suggesting that Gal-1 deficiency may be affecting B cell signaling via TLRs agonists intracellularly.

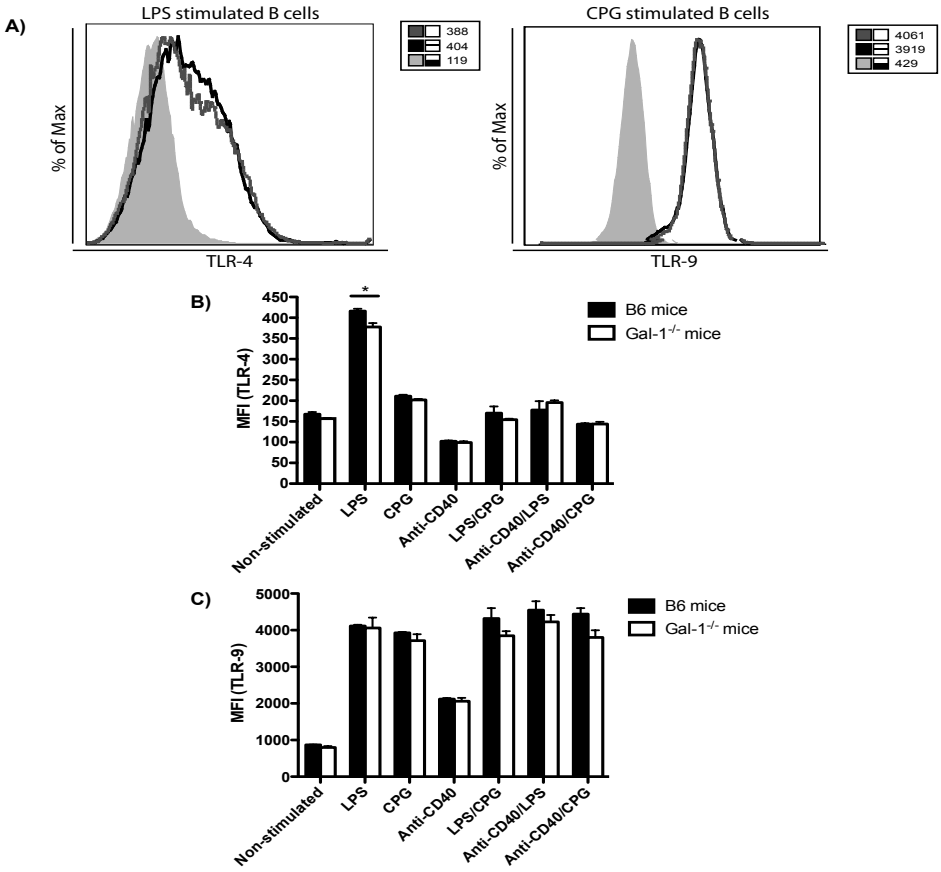

Figure 4.11 Gal-1$^{-/-}$ B cells express similar levels of TLRs as WT B cells. B cells were isolated from spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS, CPG, anti-CD40, LPS/CPG, anti-CD40/LPS and anti-CD40/CPG for 48hrs. B cells were then stained with anti-CD19, anti-TLR-4 and anti-TLR-9 (ICC). (A) Representative FACS histograms of TLR-4 and TLR-9 expression on CD19 B cells isolated from B6 (solid line) and Gal-1$^{-/-}$ (dotted line) mice (isotype is the solid grey histogram). Histograms displaying (B) TLR-4, & (C) TLR-9 expression by B cells after 48hrs. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05.

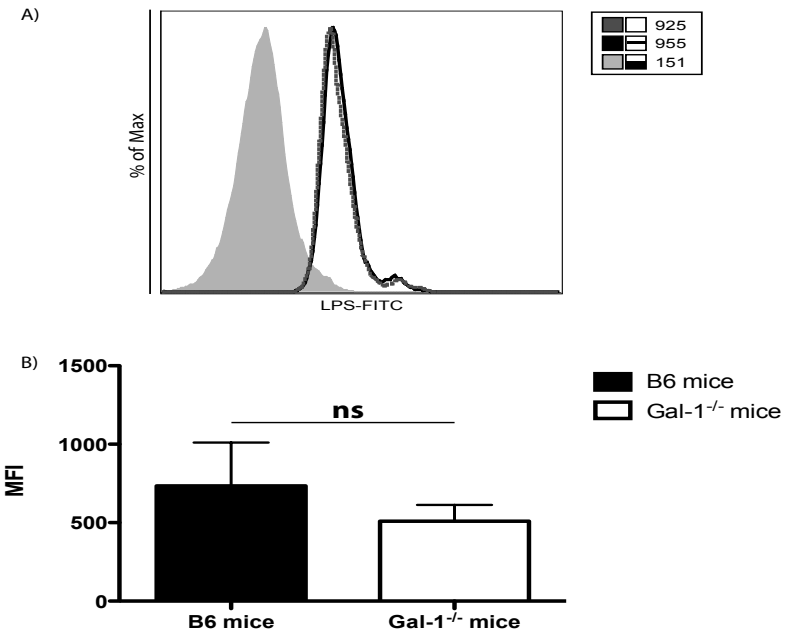

Figure 4.12 Gal-1$^{-/-}$ B cells uptake LPS at similar levels to WT B cells. Cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice, splenocytes were then incubated with anti-CD19 and FITC labeled LPS for 30mins, washed and analysed for FITC expression. (A) Representative FACS histograms of FITC labelled LPS staining of CD19 B cells isolated from B6 (solid line) and Gal-1$^{-/-}$ (dotted line) mice (non-stimulated cells are the solid grey histogram). (B) Histogram displaying mean±SEM FITC labeled LPS staining on B cells from B6 and Gal-1$^{-/-}$ mice (n=12). Statistics were calculated by t test.

4.8 Gal-1$^{-/-}$ B cells showed significant decrease in P38 phosphorylation but an increase in ERK phosphorylation in response to CPG, but not LPS.

The engagement of TLRs triggers various signaling pathways leading to the up-regulation of co-stimulatory molecules and cytokine production [217,229]. The results presented in this chapter so far have suggested that Gal-1 deficiency altered TLR-4 mediated signaling in B cells resulting in up-regulation of co-stimulatory molecules. Whereas, the engagement of TLR-9 with CPG in the absence of Gal-1 alters intracellular signaling in such a way that the kinetics of cytokines production, and in particular IL-10 production, are affected.

It has been reported that the phosphorylation of P38 and ERK, and the translocation of NF-κB are necessary steps in the signaling pathways leading to IL-10 production [300]. Therefore, I sought to determine whether Gal-1 deficiency altered intracellular signaling downstream of TLR-4 and TLR-9 by investigating the effect of TLR-ligation on P38, ERK and NF-κB molecules.

To investigate the effect of Gal-1 deficiency on intracellular signaling molecules, B cells were magnetically isolated and stimulated with CPG or LPS for 0, 10, 30, 45 and 60 minutes. Cells were then fixed and stained with anti-CD19, and then intracellularly with anti-phosphorylated p38 or anti-pERK antibodies. As a positive control for p38 and ERK phosphorylation, B cells were activated with PMA and ionomycin at each time point.

Gal-1$^{-/-}$ B cells showed a significant decrease in the percentage of cells with p38 phosphorylation compared to the WT B cells at later time points (60mins) (Figure 4.13). While stimulating B cells with LPS resulted in a slight increase in

the percentage of p38 phosphorylation in Gal-1$^{-/-}$ B cells compared to the WT B cells, in particular at the early stages of activation (10 and 30mins), but the difference was not significant (Figure 4.14).

CPG activated Gal-1$^{-/-}$ B cells, in contrast, showed a significantly higher percentage of ERK1/2 phosphorylation than WT B cells at 60 minutes (Figure 4.15). In contrast, no differences in the percentage of ERK1/2 phosphorylation between Gal-1$^{-/-}$ and WT B cells were observed when they were activated with LPS (Figure 4.16).

These results suggest that Gal-1 might act as positive regulator for P38 phosphorylation upon TLR-9 ligation, but not TLR-4. These results also suggest that Gal-1 might act as negative regulator for ERK phosphorylation upon TLR-9 ligation, but not TLR-4.

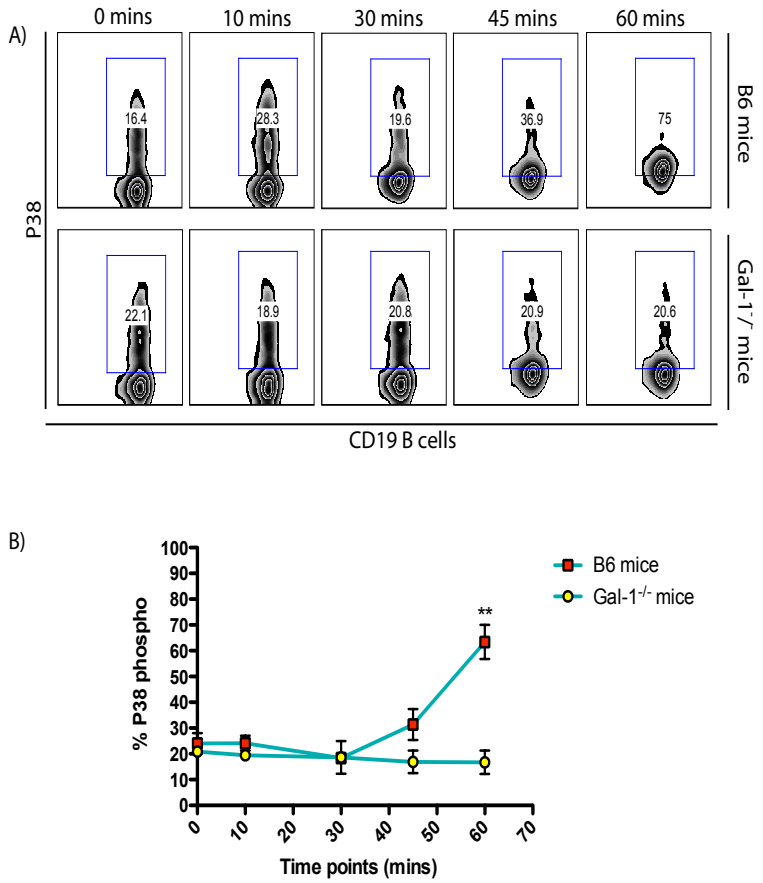

Figure 4.13 Gal-1$^{-/-}$ B cells showed a significant decrease in P38 phosphorylation in response to CPG compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with CPG for 0, 10, 30, 45 and 60mins. B cells were then fixed and stained with anti-CD19 and anti-P38 Abs (ICC). (A) Representative FACS plots of P38 phosphorylation in CD19 B cells isolated from B6 and Gal-1$^{-/-}$ mice over time course. (B) Graph displaying the percentage of P38 phosphorylation in CPG stimulated B cells isolated from B6 and Gal-1$^{-/-}$ mice over time course. Results represent mean± SEM, n=3. Statistics were calculated by t test, **P<0.005.

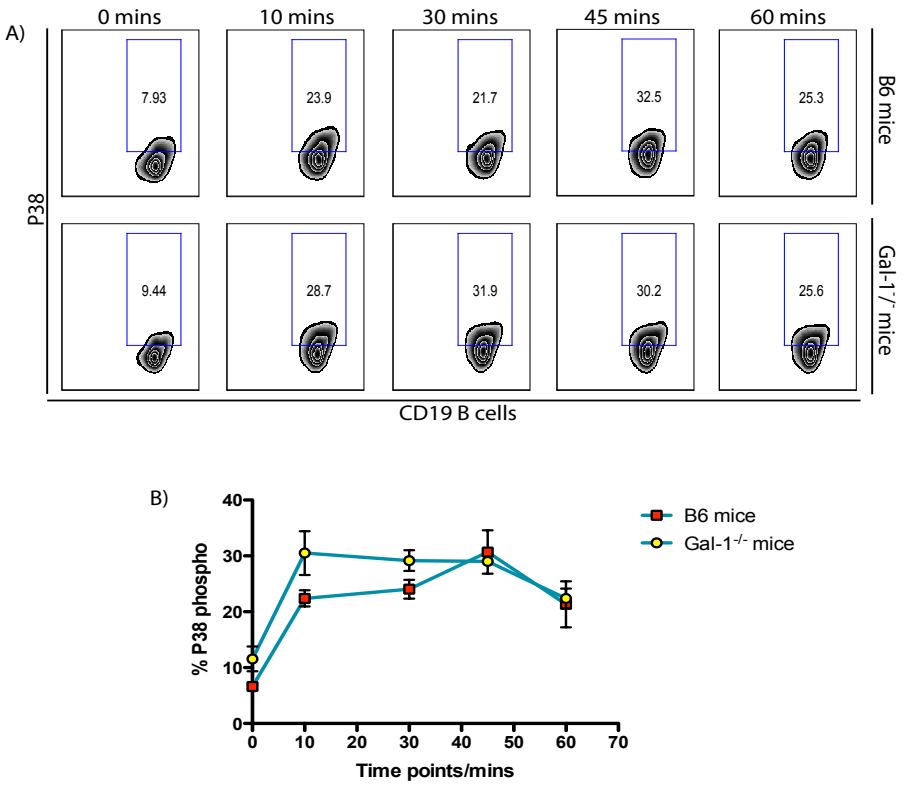

Figure 4.14 Gal-1$^{-/-}$ B cells showed no change in P38 phosphorylation in response to LPS compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS for 0, 10, 30, 45 and 60mins. B cells were then fixed and stained with anti-CD19 and anti-P38 Abs (ICC). (A) Representative FACS plots of P38 phosphorylation in CD19 B cells isolated from B6 and Gal-1$^{-/-}$ mice over time course. (B) Graph displaying the percentage of P38 phosphorylation in LPS stimulated B cells isolated from B6 & Gal-1-/- mice over time course. Results represent mean± SEM, n=3. Statistics were calculated by t test.

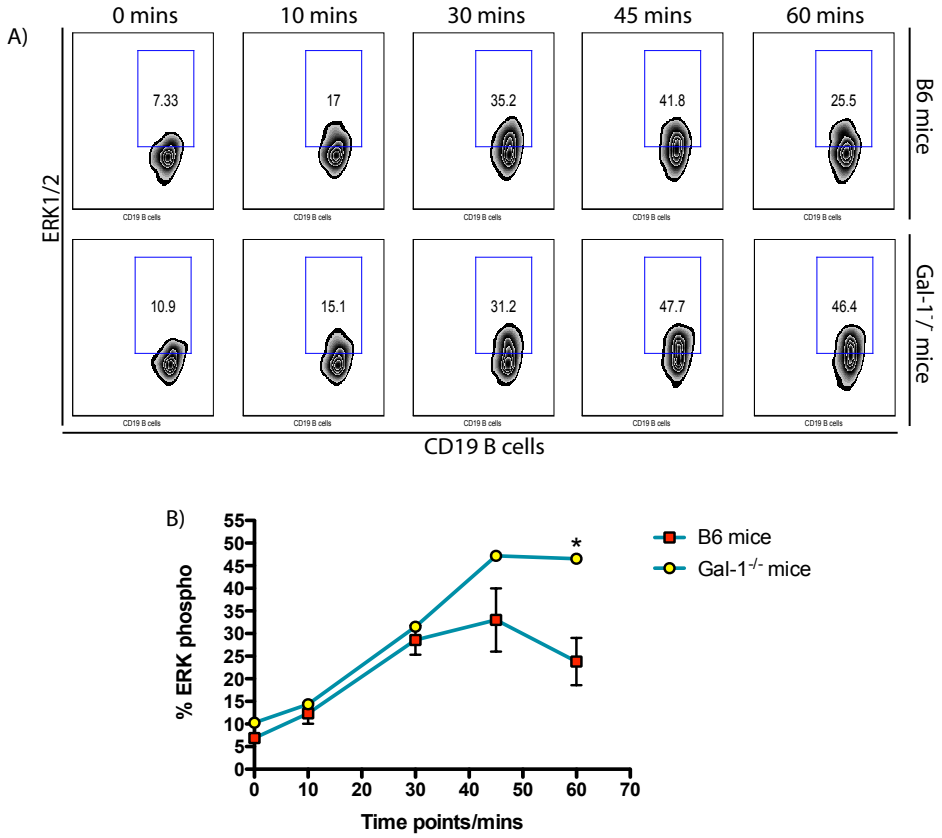

Figure 4.15 Gal-1$^{-/-}$ B cells showed a significant increase in ERK phosphorylation in response to CPG compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with CPG for 0, 10, 30, 45 and 60mins. B cells were then fixed and stained with anti-CD19 and anti-ERK Abs (ICC). (A) Representative FACS plots of ERK phosphorylation in CD19 B cells isolated from B6 and Gal-1$^{-/-}$ mice over time course. (B) Graph displaying the percentage of ERK phosphorylation in CPG stimulated B cells isolated from B6 & Gal-1$^{-/-}$ mice over time course. Results represent mean± SEM, n=3. Statistics were calculated by t test, *P<0.05.

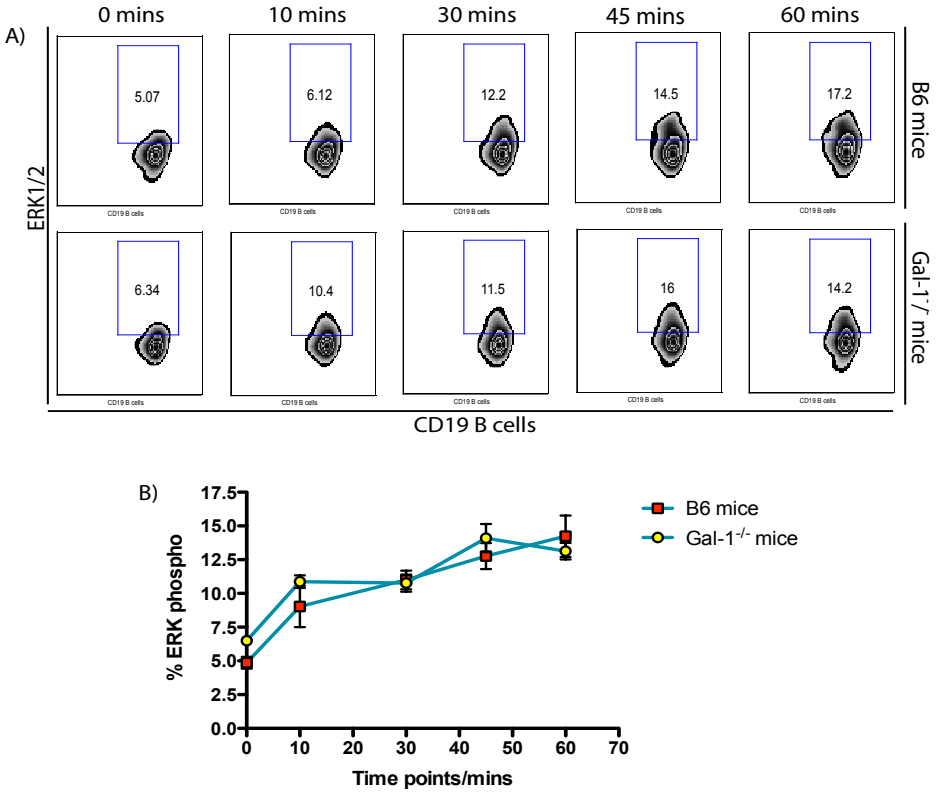

Figure 4.16 Gal-1$^{-/-}$ B cells showed no change in ERK phosphorylation in response to LPS compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS for 0, 10, 30, 45 and 60mins. B cells were then fixed and stained with anti-CD19 and anti-ERK Abs (ICC). (A) Representative FACS plots of ERK phosphorylation in CD19 B cells isolated from B6 and Gal-1$^{-/-}$ mice over time course. (B) Graph displaying the percentage of ERK phosphorylation in LPS stimulated B cells isolated from B6 & Gal-1$^{-/-}$ mice over time course. Results represent mean± SEM, n=3. Statistics were calculated by t test.

4.9 Gal-1$^{-/-}$ B cells exhibit a significant decrease in NF-κB translocation in response to both LPS and CPG.

In order to investigate whether Gal-1 deficiency altered NF-κB translocation, B cells were isolated by magnetic sorting, and activated with CPG and LPS for 0, 20, 45 and 90 minutes. Next, cells were fixed and stained with DAPI for nuclear staining and anti-NF-κB (Figure 4.17 A). Cells were then analyzed using the ImageStream flow cytometer/microscope system. The results in figure 4.17 show a significant drop in the percentage of NF-κB translocation in Gal-1$^{-/-}$ B cells compared to WT B cells following both LPS and CPG.

Collectively, the results obtained from the analysis of the signaling molecules suggest that Gal-1 might play an important role in shaping TLRs mediated signaling pathways supporting NF-κB translocation when B cells are activated through TLR-4 and 9, up-regulating P38 (TLR-9) and down-regulating ERK (TLR-9) with no influence observed on P38 and ERK in response to LPS. The alteration in the signaling pathways either affected the expression of co-stimulatory molecules (LPS) or the production of cytokines (CPG) by B cells.

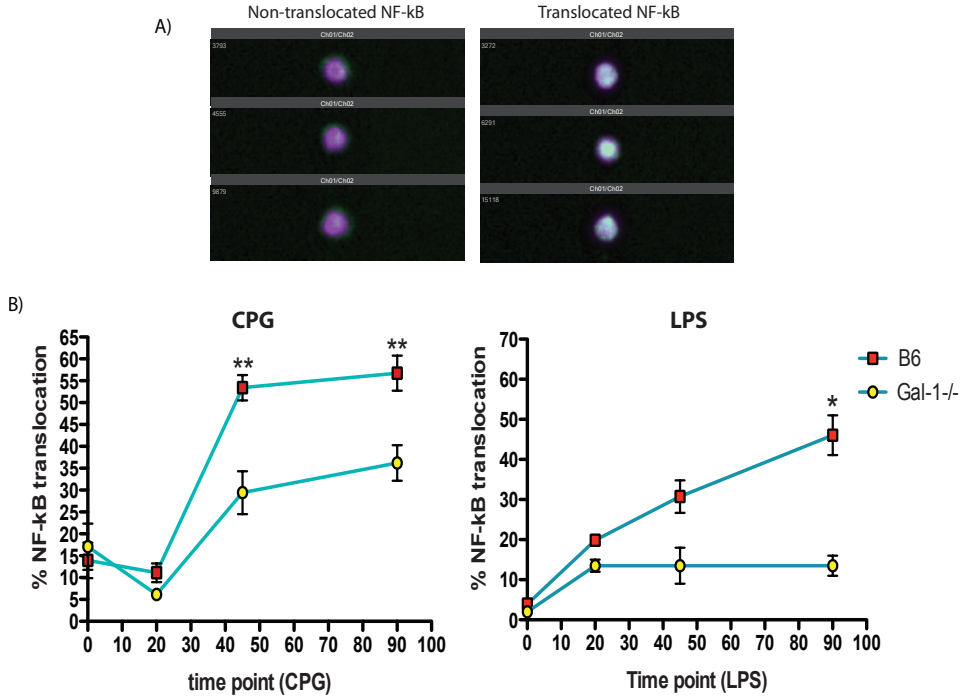

Figure 4.17 Gal-1$^{-/-}$ B cells exhibit a significant decrease in NF-κB translocation in response to both LPS and CPG compared to WT B cells. B cells were isolated form spleens of B6 & Gal-1$^{-/-}$ mice by magnetic sorting, and activated with LPS & CPG for 0, 20, 45 and 90mins. B cells were then fixed and stained with DAPI for the nucleus and anti-NF-kB (FITC) (ICC). (A) Representative ImageStream images of non-translocated NF-kB and translocated NF-kB in B cells isolated from B6 mice. (B) Graphs displaying the percentage of NF-kB translocation in CPG and LPS B cells from B6 and Gal-1$^{-/-}$ mice. Results represent mean± SEM, n=5 for CPG and 2 for LPS. Statistics were calculated by t test, *P<0.05 & **P<0.005.

Discussion

Gal-1 has been known as an immunoregulatory molecule that can regulate many cellular functions, and as an essential molecule for Treg regulatory function [187,190,191,196]. Recently, it has also been identified as a key molecule in several B cell functions [208,205,207]. In this chapter, I addressed the role of Gal-1 in B cell activation when B cells were stimulated by TLR-4 and 9 agonists, TLRs which are also known to be important in B cell responses [251]. Looking at the expression of co-stimulatory molecules and cytokine production by B cells isolated from WT and Gal-1 deficient mice, I found that Gal-$1^{-/-}$ B cells behaved differently than WT B cells. Briefly, when stimulated with CPG, Gal-$1^{-/-}$ B cells expressed significantly lower levels of IL-10, and exhibited a significant drop in NF-κB translocation and P38 phosphorylation with a significant increase in ERK phosphorylation compared to the WT B cells. Whereas Gal-$1^{-/-}$ B cells, when stimulated with LPS, expressed significantly higher levels of co-stimulatory molecules and produced significant higher amount of IL-10 compared to WT B cells and this was associated with a significant decrease in NF-κB translocation and no effect on P38 or ERK phosphorylation when compared to WT B cells.

It has previously been reported that Gal-1 has a key role in B cell development by anchoring the interaction between stromal cells, integrins, and the pre-BCR on B cells providing survival signals during their development [208]. In line with this report our results in figure 4.1B show that splenic B cell subsets isolated from Gal-$1^{-/-}$ mice have a significant increase in the percentage of T2-MZP B cells compared to the controls (WT B cells). This result suggests the possibility

that B cells might get trapped at this stage of development due to the absences of Gal-1.

Next, I examined whether the absence of Gal-1 expression by B cells affected the responses to TLR-4 and 9 stimuli. The expression of co-stimulatory molecules and cytokine production by B cells were analyzed. Gal-1 has been reported as a strong enhancer of IL-10 production, as activating PBMC with anti-CD3 antibody in the presence of Gal-1 increased IL-10 production compared to controls [204]. In addition, it has been documented that stimulating human monocyte-derived DCs cells with Gal-1 induces cell maturation and the up-regulation of CD40, CD86 and HLA-DR [301]. In the present chapter I found that Gal-1$^{-/-}$ B cells showed a significant increase in CD80, CD86, CD40 and MHC class-II molecules expressions in response to LPS, but not CPG, compared to WT B cells (Figure 4.3). In agreement with our *in vitro* results, GC B cells expressed significantly higher levels of CD80 molecules when isolated from Gal-1$^{-/-}$ mice compared to controls, and tended to express higher levels of CD86 molecules (Figure 4.4). CD80 and CD86 were selected in this experiment due to the low numbers of cells obtained from the PPs. Also, it has been previously reported that the expression of these two molecules on B cells provides essential signals for the activation and proliferation of CD4^{+} T cells [136]. CD80 and CD86 are also important for inhibiting and enhancing B cell proliferation and IgG production, respectively [291]. Collectively, these results suggest that stimulation of B cells via TLR-4 ligand in the absence of Gal-1 might results in a higher level of activation and will alter the strength of interactions with T cells via their CD80, CD86, CD40 and MHC II molecules.

On the other hand, Gal-1$^{-/-}$ B cells in response to CPG *in vitro,* showed no differences in their co-stimulatory molecule expression compared to controls, but their cytokine expression and production were significantly different compared to controls (Figure 4.6 & 4.7). Gal-1$^{-/-}$ B cells have expressed IL-10 in significantly higher levels than WT B cells at early time points post activation (Figure 4.6 B & C), however, this sharp increase was followed by a significant drop at 48 hours post stimulation (Figure 4.6 D). Suggesting that Gal-1 deficiency in B cells has accelerated the cell responses to CPG at early time points post activation to produce higher level of IL-10 which might have an autocrine affect by switching off IL-10 production at 48 hours post stimulation. In conclusion Gal-1 might act as an essential regulatory molecule for IL-10 producing B cells. My data are in agreement with a previous report by Van der leji *et al*. in which they reported that stimulating PBMCs cells in the presence of Gal-1 enhanced IL-10 production by the cells compared to a culture without Gal-1 [302]

Gal-1 has been reported to play an important role in shifting Th1/Th2 balance toward Th2 by inducing Th1 apoptosis [202,203]. Since Gal-1 has documented to regulate T cell survival, I examined under the same conditions of activation whether the absence of Gal-1 in B cells could affect their survival. As figure 4.8 shows Gal-1 deficiency did not influence B cell survival, suggesting that the results seen with IL-10 in Gal-1$^{-/-}$ B cells were not due to the viability of the cells. My data are in agreement with the previous report by Zuniga *et al*. in which they reported that Gal-1 produced by B cells activated with Trypanosoma cruzi induced T cell apoptosis, but did not affect B cell survival [207].

To determine which B cell subsets were most affected by Gal-1 deficiency, B cell subsets were purified and activated with TLR-4 and 9 stimuli, and IL-10 expression and production were measured. As with total B cells, Gal-$1^{-/-}$ T2-MZP and Gal-$1^{-/-}$ FO B cells showed the same significant drop in IL-10 expression compared to control (Figure 4.9). The data suggest that the drop seen in IL-10 production with total B cells from Gal-$1^{-/-}$ mice was due to the decrease in IL-10 observed in T2-MZP and FO B cell subsets.

The engagement of TLRs is known to activate several signaling pathways that lead to the release of cytokines and the up-regulation of co-stimulatory molecules [217,229]. It has been reported that the phosphorylation of P38 and ERK, and NF-κB translocation are important for IL-10 production [300]. After excluding that any differences that I may have observed were due to different levels of TLRs expression (Figure 4.11) or capacity to uptake LPS (Figure 4.12), NF-κB translocation, P38 and ERK phosphorylation were examined in B cells isolated from Gal-$1^{-/-}$ and WT mice. Here, I found a significant drop in NF-κB translocation in Gal-$1^{-/-}$ B cells compared to WT B cells in response to both CPG and LPS (Figure 4.17). I also observed a significant decrease in P38 phosphorylation in Gal-$1^{-/-}$ B cells in response to CPG, but not LPS (Figure 4.13 & 4.14). In contrast, a significant increase in ERK phosphorylation in Gal-$1^{-/-}$ B cells compared to WT B cells in response to CPG was seen (Figure 4.15 & 4.16). The results obtained after ligation of TLR-9 are in agreement with previous reports in which IL-10 production was associated with NF-κB down-regulation and ERK up regulation [244-248], further supporting a role for Gal-1 in regulating IL-10-production mediated by TLR-9 signalling. However, in the

case of TLR-4 ligation, the high IL-10 production was only correlated with the decrease in NF-κB translocation, but no obvious involvement of ERK or P38 molecules.

Altogether the findings in this chapter suggest that Gal-1 plays different regulatory roles in controlling IL-10 production by activated B cells depending on the TLR that has been stimulated. However, how Gal-1 provides this role and where Gal-1 is involved in these pathways need to be further investigated. For example, immunoprecipitation can clarify at which stage Gal-1 is involved.

Moreover, GaL-$1^{-/-}$ B cells activated via TLR-4 showed up regulation in co-stimulatory molecules. However, none of the signalling pathways that have been investigated in this chapter showed an association, suggesting the possibility that other pathways might be involved, such as JNK pathway.

In summary, I have reported in this chapter for the first time that Gal-1 has an important role in B cell responses to TLR-4 and TLR-9, and that the absences of Gal-1 has affected B cells intracellular signalling pathways to up-regulate both their co-stimulatory molecules and IL-10 production in response to LPS, while affecting only IL-10 production in response to CPG. The findings in this part of my thesis contribute to the understanding of the central role that Gal-1 plays in regulating B cells functions in response to TLR ligation. This builds on the results in chapter 3 were Gal-1 was found to play an essential role in the regulatory function of B cells.

Chapter 5- Results 3

Chapter 5

There is growing evidence that B cells contribute to the maintenance of transplant tolerance [303,304], as well as promoting chronic allograft responses [305,306,307]. In mice, anti-CD20 depletion accelerates skin graft rejection [305], anti-CD45RB induced tolerance to heart transplants requires the presence of B cells [308], and resting B cells expressing donor antigen have been observed to indefinitely prolong heterotopic heart transplant survival [309]. In rats, long-term allograft tolerance is characterized by an accumulation of B cells expressing genes such as BANK-1 that are associated with tolerance [310].

B cells have been primarily thought of as the source of alloantibodies that activate complement and varieties of effector cells, and mediate rejection [63]. For instance depletion of B cells with Rituximab in ABO incompatible renal transplant recipients has had positive results in delaying rejection [311,312]. However, the use of B cell depletion as induction therapy for some kidney transplant patients has lead to increased incidence of acute cellular rejection [60]. It is possible that in these cases B cell depletion is removing B cells that contribute to tolerance induction rather than those promoting rejection [61].

A “B cell signature” has been documented in spontaneously tolerant patients who received HLA mismatched kidneys and are not taking immunosuppreive agents. Genes associated with B cell function were up-regulated in PBMCs of tolerant patients compared to healthy controls and non-tolerant recipients [55]. In addition, the proportions of transitional B cells in the patients peripheral blood

were reported to be higher in drug free tolerant patients compared to healthy individuals and non-tolerant controls [56].

In this chapter a mouse model of skin transplant was used to investigate the tolerogenic role of B cells in transplantation. In line with the findings in humans, T2-MZP B cells were expanded in mice tolerised by donor splenocyte transfusion (DST) and anti-CD40L (MR1) treatment. Upon adoptive transfer, T2-MZP B cells isolated from DST + MR1 treated mice were able to prolong skin graft survival *in vivo* and suppress TNF-α expressing T cells *in vitro*. Although in this model there was no evidence for T2-MPZ B cell IL-10 production, T2-MZP B cells isolated from DST + MR1 treated mice up-regulated TIM-1 expression but lose expression of CD86 suggesting an alteration in their T cell stimulatory capacity.

Note all the experiments in this chapter were done in the SPF facilities of the animal house, and most of the experiments were done together with 2 post-docs.

5.1 B cells are required for long-term tolerance to MHC I mismatched skin grafts in an experimental model of transplant tolerance.

To investigate whether the B cells that are associated with transplant survival represent regulatory B cells that may contribute to the maintenance of tolerance we returned to a mouse model of experimental skin graft tolerance. Tolerance to skin grafts can be rendered in B6 mice receiving B6-K^d skin by pre-treatment with B6-K^d splenocytes and anti-CD40L antibody (MR1) [313]. Seven days before receiving a skin graft mice received $25x10^6$ donor splenocytes (DST) + 250 μg MR1 plus an additional injections of 100μg of MR1 three days before the graft, on the day of graft and four days subsequently. In order to determine whether the establishment of DST+ MR1 induced tolerance required B cells we attempted to induce tolerance in B cell deficient mice. Seven days prior to B6-K^d skin graft, B6 and μMt (B cells deficient) mice were treated with DST+MR1 (Figure 5.1 A). Allografts survived indefinitely on B6 recipients. Although the treatment of μMt mice did prolong allograft survival compared to untreated and MR1 alone treated controls, tolerance was not seen in μMt mice treated with DST+MR1 (Figure 5.1 A). This result suggests that B cells play a role in maintaining tolerance to allograft. Interestingly, although the level of alloantibody was reduced in μMt mice treated with DST+MR1 compared to the controls, there was no correlation between graft rejection and alloantibody IgG production (Figure 5.1 B). Untreated mice produced detectable levels of anti-K^d antibody in their serum at one month after transplant. However, although most of the mice that received MR1 treatment alone had rejected the skin graft, none of the mice had detectable level of anti-K^d IgG antibody in their serum (Figure

5.1 B). CD40/CD40L interactions are essential for B cell class switching and generation of an antibody responses, it is possible that the MR1 treatment blocks this process [314]. Comparison of untreated and MR1 treated mice suggests that graft rejection of equal speed can occur in this model without the presence of IgG alloantibody production (Figures 5.1 A & B).

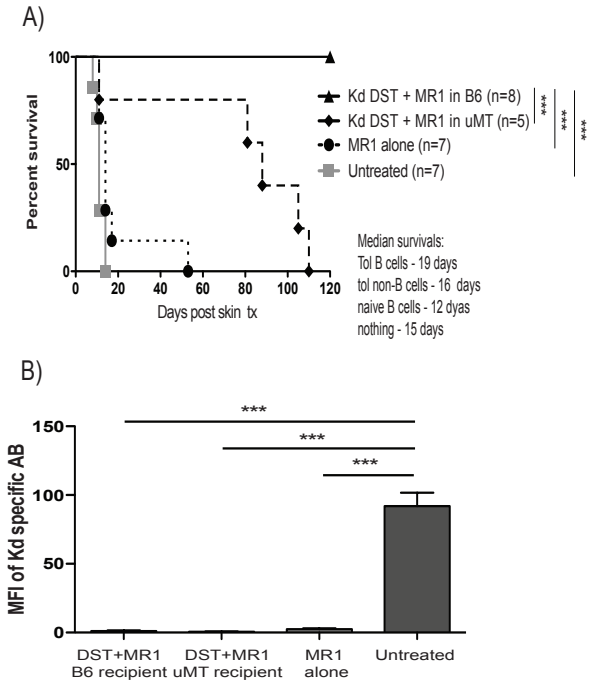

Figure 5.1 B cells are required for long term DST + MR1 mediated tolerance to MHC I mismatched skin grafts. B6 and μMt mice received B6 K^d splenocytes 7days prior to a B6 K^d skin graft in parallel of a MR1 treatment. As controls, B6 mice received B6 splenocytes 7days prior the graft or received MR1 treatment and B6 K^d skin grafts. (A) Survival curves display skin graft survival (n≥5); Log Rank (Mantel-Cox) test. Anti-K^d IgG production was measured in the blood of mice in (A) by FACS at day30 post-transplant. (B) Bar graph displaying mean + SEM MFI of Anti-K^d specific IgG (n=5).*** p≤0.001, one way ANOVA and Bonferroni's multiple comparison test.

5.2 T2-MZP and MZ B cells are transiently increased in tolerised mice.

It has previously documented that B cells with T2-MZP phenotype have a regulatory role in mouse model of SLE and arthritis [146,149], MZ B cells in CIA [144], and B10 ($CD5^+$ & $CD1d^{high}$) B cells in EAE [168]. Therefore, we sought to determine if there were phenotypic changes in the B cell pool in tolerant mice compared to rejecting mice over the course of rejection. B6 mice were tolerised to B6 K^d skin grafts as above and spleens were recovered at day 9, 12, 15, 18 and 21 mice after B6 K^d skin graft. Control mice received $25x10^6$ B6 splenocytes in order to allow for alterations in B cell numbers due to the DST in the tolerisation protocol. Subsequently, B cell subsets, B10 B cells and iNKT cells were phenotyped in the spleens obtained from each set. T2-MZP and MZ B cells showed significant increases as percentages of total splenic B cells in tolerised mice compared to rejecting mice at day 12 (Figure 5.2 A & B). B10 B cells showed a trend for an increase in tolerised mice compared to rejecting mice, however, this increase was not significant (Figure 5.2 E & F). FO and T1 B cells showed no changes in their proportions (Figure 5.2 C and D). iNKT cells, also showed no differences in their percentage (Figure 5.2 G & H).

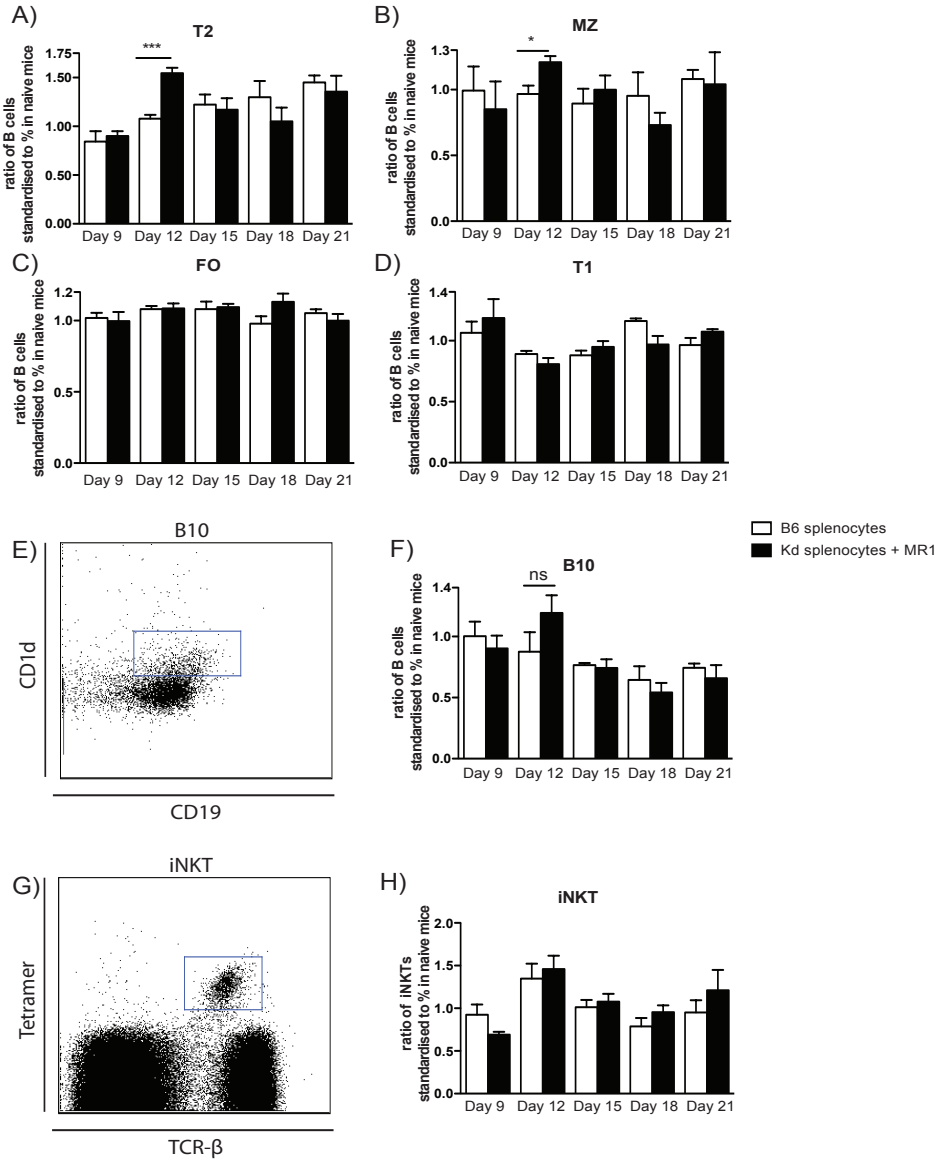

Figure 5.2 An increased percentage of T2-MZP and MZ B cells in the spleens of B6.K^{d} DST + MR1 tolerised mice 12 days after B6.K^{d} skin graft. B6 mice were tolerised to B6.K^{d} skin as described in Figure 1. Control mice received B6 splenocytes 7 days prior to B6.K^{d} skin grafts to allow for changes in B cell numbers due to DST. Spleens were recovered at marked days post-graft and the percentages of CD19^{+}CD21$^{neg/low}$CD24^{+} T1, CD19^{+}CD21^{low}CD24^{low} FO, CD19^{+}CD21^{high}CD24^{high}CD23^{low} MZ, CD19^{+}CD21^{high}CD24^{high}CD23^{high} T2-MZP, CD19^{+}CD1d^{+}CD5^{+} B10 B cells, and TCR-β^{+}CD1d loaded tetramer^{+} iNKT cells were determined by FACS staining. (E and G) Representative FACS plots showing gating for B10 B cells and iNKT cell. (A-D, F and H) The percentages of marked cell subsets in tolerised and control mice standardized to the percentages in naïve mice at the different days following graft. Percentages analysed by one-way ANOVA and Bonferroni's multiple comparison test. *** p<0.001, * p<0.05. n ≥3 for each group for each time point.

5.3 Adoptive transfer of T2-MZP B cells isolated from tolerised mice can prolong MHC I mismatched skin graft survival.

Since T2-MPZ and MZ B cells displayed a significant increase in their proportions in the spleens of tolerant mice compared to controls, we examined their potential as immuneoregulatory cells in inducing transplant tolerance. Here, tolerance to B6 K^d skin grafts was induced in B6 mice as discussed in the previous section, B cell subsets were purified from splenic B cells of either tolerised or naïve B6 mice by FACS 14 days post transplant, and $1x10^6$ cells of each subset were adoptively transferred (IV) into naïve B6 mice. One day subsequently the B6 mice received B6 K^d skin transplants. CD8 cells were depleted by IP injection of anti-CD8 antibody on day -1, 0 (day of skin graft), +1 and every 7 days following the skin graft, and serums were collected 30days post transplant. In this model, T2-MZP B cells where the only subset to significantly prolong the skin survival (mean 19 days) compared to CD8 only controls (mean 15 days) or mice receiving any other B cell subset (means 12, 15.5 and 15 days for T1, FO and MZ, respectively) (Figure 5.3 A). Consistent with my previous observation in chapter 3, T2-MZP B cells isolated from naïve B6 mice did not prolong allograft survival compared to the controls (Figure 5.3 B). This suggest that T2-MZP B cells needs to be activated *in vivo* to exert their regulatory function.

In line with the results in figure 5.1 there was no correlation between the levels of alloantibody and allograft rejection (Figure 5.3 C). Although T2-MZP B cells from tolerant mice prolong allograft survival and reduced the level of alloantibody, T1 and MZ B cells also showed low levels of alloantibody in their

serum even though they did not prolong skin survival following adoptive transfer (Figure 5.3 C). Suggesting that alloantibody production is not the driving force behind rejection in this model.

It has been shown in autoimmune models that T2-MZP B cells are capable of suppressing TNF-α^+ T cells *in vitro*. Therefore, we examine whether T2-MZP B cells isolated from tolerant B6 mice can exhibit the same affect in our model. In this experiment, B cells were isolated from the spleens of tolerised B6 mice by magnetic sorting. B cell subsets were then FACS sorted, and co-cultured with CD3/CD28 beads activated $CD4^+$ T cells (1:1) isolated from mice that had rejected B6 K^d skin graft for 48 hours. PMA, Ionomycin and brefeldin A were added for the last 4 hours of culture. TNF-α was measured by intracellular staining and flow cytometry. The results showed that T2-MZP B cells were able to significantly suppress CD3/CD28 beads activated $CD4^+$ T cells TNF-α expression by around 25% (Figure 5.4 A & B). In this model, no other B cell subset displayed a statistically significant inhibition of TNF-α expression by CD3/CD28 beads activated $CD4^+$ T cells (Figure 5.4 A & B).

In summary, only T2-MZP B cells prolonged skin survival *in vivo* and suppressed CD3/CD28 activated $CD4^+$ T cell TNF-α expression *in vitro* when isolated from tolerised, but not naïve B6 mice. Moreover, alloantibody production seems not to be correlated with skin rejection in this model.

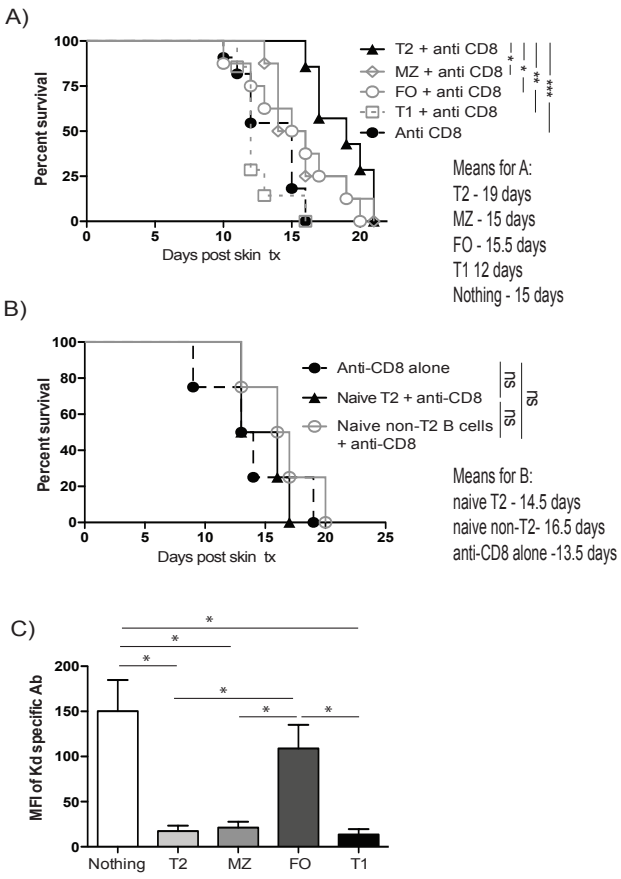

Figure 5.3 Adoptive transfer of T2-MZP B cells isolated from the spleens of B6.K^d DST + MR1 tolerised mice can prolong B6.K^d skin graft survival. (A) CD8 depleted naïve B6 mice received 1x10^6 splenic T1, T2-MZP, MZ, or FO B cells from B6 mice tolerised to B6.K^d skin by DST + MR1. One day later mice received B6.K^d skin grafts. Survival curves show skin graft survival (n≥7). Median survivals (days): anti-CD8 alone-15, T2-MZP-19, MZ-15, FO-15.5, and T1-12. (B) CD8 depleted naïve B6 mice received 1x10^6 splenic T2-MZP B cells or pulled non-T2-MZP B cells from naïve B6 mice (n=4). One day later mice received B6.K^d skin grafts. Survival curves show skin graft survival. Median survivals (days): anti-CD8 alone-13.5, naïve T2-MZP-14.5, naïve non-T2-MZP-16.5. (C) Anti-K^d IgG production was measured in the blood of mice in (A) by FACS at day 30 post-transplant and is represented as described in Figure 1B (n=5). Survival curves analysed by Log Rank (Mantel-Cox) Test, antibodies analysed by one-way ANOVA and Bonferroni's multiple comparison test. *** p<0.001, * p<0.05. ns: not significant.

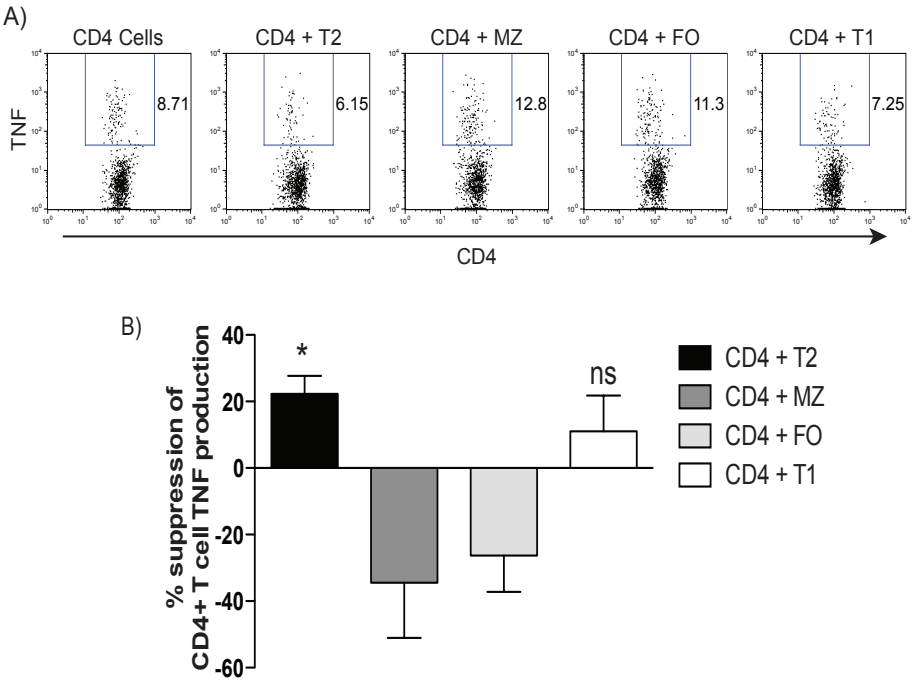

Figure 5.4 T2-MZP B cells isolated from tolerised mice downregulate suppress T cell TNF α expression. B cell subsets were purified from B6.K^{d} tolerised mice (day14 post-graft). Splenic naïve CD4^{+} T cells were stimulated with anti-CD3/anti-CD28 antibodies for 48hrs ± the different B cells subsets (ratio 1:1). PMA, Ionomycin and Brefeldin A were added for the last 4hrs of culture. TNFα expression was detected by intracellular staining. (A) Representative FACS plots of T cell TNFα expression. (B) Bar charts depict mean suppression by each B cell subset +SEM. Statistics were calculated by one-way ANOVA and a Dunnett post test comparing TNFα expression in co-cultures with expression by T cells cultured alone. (n=4). * $p<0.05$, ns: not significant.

5.4 The suppressive capacity of T2-MZP B cells isolated from tolerant mice is not related to IL-10 or TIM-1 expression.

In order to understand the possible mechanisms behind the ability of T2-MZP B cells isolated from tolerant mice to prolong allograft survival and suppress TNF-α expressing $CD4^+$ T cells *in vitro* we examined the expression of several molecules that have previously been reported to play a role in regulatory B cells function or B cells tolerance, such as IL-10, CD80/CD86, and TIM-1.

IL-10 has been documented to play the major role in T2-MZP B cells regulatory function in autoimmunity [146,149]. Recently, in transplantation, it has been reported that TIM-1^+ B cells have the capacity to prolong islet allograft survival post to their adoptive transfer [173]. Moreover, the expression of B7 molecules has been associated with B cells ability to suppress several autoimmune diseases [168]. Therefore, we examined whether the expression of any of these molecules could be involved in T2-MZP B cells suppressive capacity.

To investigate the role of IL-10, B6 mice tolerised to B6 K^d skin and control non-tolerised B6 mice were sacrificed at days 9, 12, 15, 18 and 21. Subsequently, B cells were magnetically isolated from the spleens of tolerant and control mice and sorted by FACS into subsets. The levels of IL-10 mRNA transcription by purified B cell subsets from tolerised mice were tested by RT-PCR and compared to the same subsets purified from naïve mice. There was a trend for IL-10 transcription to be up-regulated in T2-MZP B cells isolated from tolerant mice at day 15 post transplant, but this increase was not significant (Figure 5.5 A). T1 B cells, which did not prolong skin allograft survival upon adoptive transfer, however did significantly upregulate IL-10 transcription over the course

of the study (Figure 5.5 A). Furthermore, we investigated whether *in vitro* re-stimulation of sorted T2-MZP B cells isolated from naïve and tolerised mice could highlight a difference in their IL-10 expression. Here, B cells were magnetically isolated from the spleens of tolerant and naïve B6 mice and sorted by FACS into subsets, and re-stimulated *in vitro* with a combinations of LPS, CPG, and anti-CD40 for 48 hours. After 48 hours of culture, supernatants were collected for ELISA. T2-MZP B cells show no difference in the capacity to secrete IL-10 between those obtained from naïve and tolerant mice (Figure 5.5 B). These results suggest that IL-10 production is not associated with the ability of T2-MZP B cells (isolated from tolerant mice) to prolong allograft survival.

Besides IL-10 expression, we also investigated the expression of CD80, CD86 and TIM-1 on T2-MZP B cells isolated from tolerant mice. Here, B cells were magnetically purified from the spleens of either from B6 mice tolerised to B6 K^d skin graft at day 14 post-transplant, or from naïve B6 mice. The expression of CD80, CD86 and TIM-1 on B cell subsets was then determined by flow cytometry. T2-MZP B cells isolated from tolerant mice showed no differences in CD80 expression compared to control (naïve mice), however CD86 expression was significantly decreased in T2-MZP B cells from tolerant mice compared to naive T2-MZP B cells (Figure 5.5 C & D). On the other hand, the percentage of T2-MZP B cells expressing TIM-1 was significantly higher among T2-MZP B cells isolated from tolerant mice compared to naïve mice (Figure 5.5 E). However, TIM-1 was also significantly increased within MZ B cells, and showed a trend of higher expression in FO and T1 subsets (B cell subsets that do not

prolong allograft survival) (Figure 5.5 E). This would suggest that TIM-1 is not a marker of regulatory B cells in this model.

In summary, we report for the first time an experimental mouse model of transplant tolerance that is associated with an increase in T2-MZP B cells. We demonstrated that adoptive transfer of these B cells was able to prolong skin graft survival suggesting a direct regulatory role for this subset in transplantation. In contrast to T2-MZP B cells isolated from tolerised mice, T2-MZP B cells isolated from naïve mice were not able to prolong graft survival. Finally T2-MZP B cells isolated from tolerised mice downregulate B7 co-stimulatory molecules, suppress T cell TNFα production *in vitro*.

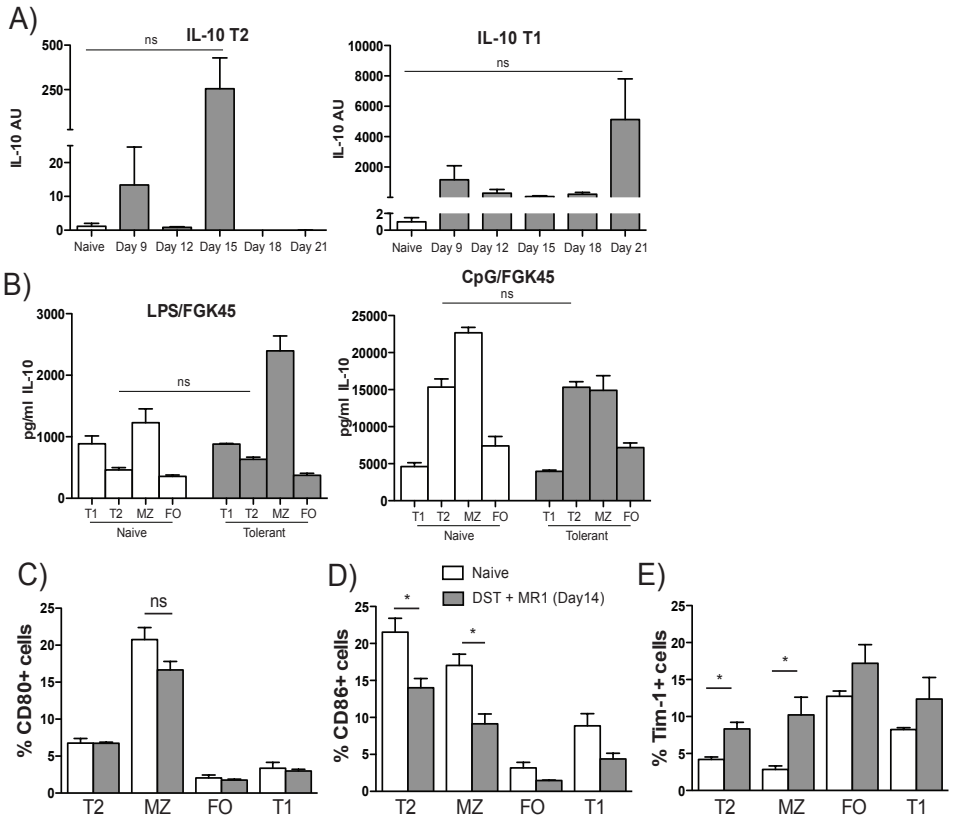

Figure 5.5 T2-MZP B cells from B6.K^{d} DST + MR1 tolerised B6 mice do not upregulate the expression of IL-10 or Tim-1^{+}, but downregulate B7 expression *in vivo*. (A) Quantitative PCRs for IL-10 were performed on cDNA purified from T1, T2-MZP, MZ and FO B cells isolated from tolerised mice on marked days. Graphs show the relative expression between IL-10 and actin as calculated by the $2^{-\Delta\Delta Ct}$ method. (B) T1, T2-MZP, MZ and FO B cells were purified from B6.K^{d} tolerised mice at day14 following skin graft or from naïve mice. B cells were stimulated with FGK45 and LPS, or FGK45 and CpG ODN1826 for 48hrs. IL-10 concentrations in supernatants were assessed by ELISA. Graphs show mean + SEM of IL-10 concentration. (n=4). (C and D) Splenocytes from naïve and B6.K^{d} tolerised mice (day14 post-graft) were stained for B cell subsets and (C) CD80 and (D) CD86 expression. Bar charts show mean percentage expression + SEM. Statistics were calculated by one-way ANOVA and Bonferroni's multiple comparison test. (n=4). (E) Splenocytes from naïve mice and B6.K^{d} tolerised mice, at day14 post skin graft, were stained for B cells and Tim-1 expression. Graphs show mean % expression + SEM. (n=4). Statistics calculated by one-way ANOVA and Dunnetts post test (A) or Bonferroni's multiple comparison test. * $p<0.05$, ns: not significant.

Discussion

In humans, maintenance of an immature transitional B cell population has been associated with both spontaneous tolerance to renal transplants as well as remission from systemic lupus erythematosus following B cell depletion [55,56,315]. Here we report for the first time an experimental mouse model of transplant tolerance that is similarly associated with an increase in T2-MZP B cells. We demonstrate that adoptive transfer of these B cells is able to prolong skin graft survival suggesting a direct regulatory role for this subset in transplantation. In contrast to T2-MZP B cells isolated from tolerised mice, T2-MZP B cells isolated from naïve mice were not able to prolong graft survival. Finally T2-MZP isolated from tolerised mice down-regulated B7 co-stimulatory molecules, and suppressed T cell TNFα production *in vitro*.

In the present chapter, we have shown that transplant tolerance is induced via DST plus MR1 only in B6 mice, but not uMT mice or B6 mice treated with MR1 alone (Figure 5.1 A). Here, the level of allo-IgG antibodies in the serums of both allograft recipients B6 and uMT mice that was treated with DST plus MR1 show the same significant drop compared to controls (Figure 5.1 B). These results suggest that B cells are necessary for the establishment of DST + MR1 induced tolerance, and that no absolute correlation between graft rejection and IgG alloantibody production as none of the mice receiving MR1 treatment alone had detectable serum anti-K^d IgG even though they had rejected the graft. This result supports the findings of Sagoo et al and Newell et al. in man [55,56]. They found that the difference between tolerant and non-tolerant transplant recipients was in the number of B cells in the periphery and the expression of

genes that encoded for the variable region segment of the Ig light chain. Therefore, they called it “B-cell signature” and suggested that these genes could help in selecting transplant recipients who should receive immunosuppressive, from those who should not [55,56]. Although B cells have been primarily thought of as the source of alloantibodies that activate complement and varieties of effector cells [281], the presence of antibodies may simply be an indicator for B cell involvement in antibody-independent mechanisms [66]. This result also agrees with published data that B cells do not influence the immune response solely through the production of antibodies, but also via cytokine production or by presenting antigens [66].

In lupus model, it was observed that T2-MZP B cells absolute number was decreased during the disease [149]. Here, a significant expansion only in splenic T2-MZP and MZ B cells, but not FO or T1 B cells, was observed in mice that have been rendered tolerant via DST + MR1 treatment (Figure 5.2). Furthermore, T2-MZP B cells, but not MZ, T1 or FO B cells, isolated from tolerised mice at day 12 prolonged allograft survivals (Figure 5.3 A). This result confirms previous reports in autoimmunity, in which the adoptive transfer of B cells with T2-MZP B cells phenotype have protected from the disease [146,149]. However, in our model only T2-MZP B cells isolated from tolerised mice improved the allograft skin survival, but not the same subset when isolated from naïve mice (Figure 5.3 B). This result suggest that tolerising the mice with DST + MR1 has primed T2-MZP B cells *in vivo* to exert their regulatory function. It is possible that tolerising the mice via anti-CD40 ligand mAbs and donor splenocytes transfusion has produced activated tolerogenic T cells or Tregs that

interact and activate B cells in particular T2-MZP B cells to exert their regulatory function. This hypothesis can be confirmed by isolating T cells and Tregs from tolerised mice and co-culturing them with B cell subsets isolated from naïve B6 mice, and studying how could the interaction between these cells could functionally influence the B cells, in particular, T2-MZP B cells, by re-culturing the induced B cell subsets with allo-DCs activated $CD4^+$ T cells.

Interestingly, although adoptive transfer of tolerised T2-MZP B cells does reduce the level of alloantibody detectable in the serum of graft recipients compared to controls, there was not a correlation between graft rejection and alloantibody production (Figure 5.3). Although prolongation of graft survival was observed only when mice were treated with T2-MZP B cells we found that alloantibody was reduced in the serum of mice treated with T2-MZP, MZ or T1 B cells (figures 5.3 A & C). While in all cases where high levels of alloantibody are produced we see rejection, the converse is not true; we see rejection in the absence, or with low levels, of alloantibody. Thus, alloantibody production may not be the driving force behind rejection in this model. Alternatively alloantibody production may be more sensitive to regulation and both T1 and MZ B cells isolated from tolerised mice may possess some regulatory capacity.

Beside the ability of T2-MZP B cells isolated from tolerised mice to prolong the allograft skin survival, they were the only B cell subset capable of inhibiting TNF-α expressing $CD4^+$ T cells *in vitro* (Figure 5.4). Confirming the regulatory role that T2-MZP B cells can provide.

In terms of regulatory function most previous studies on Breg cells in mice and humans have focused on their ability to produce IL-10 [256,257]. Moreover, IL-

10 expression was necessary for TIM-1$^+$ B cells to prolong MHC mismatched islet allograft survival [173, 316]. Here, we examined the expression of IL-10 mRNA in B cell subsets isolated from tolerised mice at different time points post transplant, as well as IL-10 production by (ELISA) B cell subsets isolated from tolerised mice and activated with LPS plus anti-CD40 mAbs and CPG plus anti-CD40 mAbs. The results showed that there is no significant increase in the expression of IL-10 mRNA in T2-MZP B cells at any time point (Figure 5.5 A). In addition, there was no significant difference in IL-10 production between any of the *in vitro* activated T2-MZP B cells isolated from tolerant and naïve mice (Figure 5.5 B). Finally, the finding that tolerised T1 B cells significantly upregulated IL-10 mRNA transcription compared to naïve T1 B cells further suggests IL-10 is not primarily responsible for prolongation of graft survival as adoptive transfer of T1 B cells was unable to delay rejection (Figure 5.3 A). However, although IL-10 production has defined Breg cells in the field of autoimmunity it is not as definitively associated with tolerance in investigations of transplantation [56,55,317,318]. Studies that have reported an association of B cells with tolerance to kidney transplants in humans did not report an association with IL-10 mRNA transcription [56,55,317]. In mice, studies of the capacity of anti-CD45RB monotherapy to induce tolerance to cardiac allografts reported that, while this tolerance protocol is dependent on the presence of B cells, IL-10 production by B cells was in fact counter-regulatory and IL-10 neutralization improved graft outcome [318]. The role of IL-10 in B cell function in our transplant model needs to be adequately elucidated. Adoptive transfer of

IL-10$^{-/-}$ T2-MZP B cells tolerised *in vivo* with DST + MR1 into a second allograft recipient mice can completely rule out the role of IL-10 in our model.

To explore other possible mechanisms involved in the protective role of T2-MZP B cells, we studied the expression of CD80, CD86 and TIM-1 on B cell subsets isolated from the spleens of tolerised and naïve mice at day 14 post transplant. There were no significant differences in the expression of CD80 molecules on T2-MZP B cells isolated from naïve or tolerised mice (Figure 5.5 C). However, Figure 5.5 D show that there was a significant drop in CD86 molecule on T2-MZP B cells isolated from tolerised mice compared to those isolated from naïve mice (Figure 5.5 D). It is well known that B cells deliver essential signals for the activation and proliferation of both CD4 T cells and memory T cells through CD80 and CD86 [134,135,136]. An experiment that was performed using mice that lack the expression of CD80 and CD86 on B cells, in an arthritis model, demonstrated that the expression of CD80 and CD86 by B cells is needed for auto-reactive T cell proliferation and activation [136]. *In vitro,* re-activation of CD4^{+} T cells obtained from mice that express B cells deficient of CD80/CD86 molecules with cartilage antigen resulted in less proliferation in CD4^{+} T cells compared to the WT CD4^{+} T cells [136]. The fact that CD86 binds with low affinity to CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) and with high affinity to CD28 [291] suggest that the significant drop in CD86 molecules expression on T2-MZP B cells compared to control has in turn affected the level of T cell activation. It might be helpful to test this theory by the adoptive transfer of CD86$^{-/-}$ T2-MZP B cells isolated for tolerised mice into all-graft skin recipients.

In the context of transplantation, TIM-1$^+$ B cells, generated following treatment of mice with a low affinity/blocking anti-TIM-1 antibody, have recently been described to be able to prolong MHC mismatched islet allograft survival following adoptive transfer [173]. It is not clear whether TIM-1 is associated with Breg function in our model. We investigated the expression of TIM-1 on B cells isolated from DST + MR1 tolerised mice. We found that TIM-1 was expressed across all tolerised B cell subsets and, while it was upregulated on T2 B cells following tolerisation, there was still a lower percentage of TIM-1$^+$ T2 than any other subset (Figure 5.5 E). In a recent paper, Qian *et al*. have reported an induction of Breg cells by regulatory dendritic cells and similarly to us they have not found an increase in TIM-1 expression by the B cells [319]. These results may suggest that there are a number of possible Breg cell phenotypes each specific for a particular mouse models.

In summary, we have demonstrated that DST + MR1 tolerised T2-MZP B cells have a direct effect on CD4$^+$ T cell activation *in vitro* and *in vivo*. We hypothesize that MR1 + DST activates and expands T2-MZP B cells that in the context of diminished CD86 costimulation, prevent allospecific T cell activation. The B cell signature, and preserved T2-MZP B cell compartment, of spontaneously tolerant kidney transplant patients may represent a similar process.

Conclusion

In this thesis, I have shown that B cells exert their regulatory role in prolonging skin transplant survival only when they were educated and primed *in vivo*. The experiments in this thesis have shown that B cells, in particular T2-MZP B cells have an inhibitory effect *in vivo* and *in vitro* either after being isolated from tolerised mice or obtained from mice kept under non-hygienic conditions. The exact mechanisms that lead to this priming are unclear, and whether the same mechanisms apply for both conditions is also not clear. Different possibilities for each situation have been discussed at the end of the results chapter. Although the two systems that have been used in this thesis leads to the same results, where B cells suppressed TNF-α expressing $CD4^+$ T cells *in vitro* and prolonged skin graft survivals, the process of priming T2-MZP B cells *in vivo* is different. T2-MZP B cells isolated from mice kept under non-hygienic conditions might be primed through their interaction with gut microbiota via engagement of TLRs. In the mice rendered tolerant, T2-MZP B cells may be induced to have a regulatory function by interacting in the tolerant mice with regulatory T cells. However, further work is necessary to understand the differences between the two ways that Bregs are induced. An interesting preliminary result has shown that B cells obtained from Gal-$1^{-/-}$ mice rendered tolerant using MR1 and DST are suppressive, further emphasizing the difference between the two ways in which B cells can be induced to become regulatory.

The important role that hygiene plays in inducing B cell regulatory function brings the CV mouse model described here closest to the situation in man. Also, my results suggest that T2-MZP B cells are regulatory in transplantation,

thus one of the reasons rituximab may have resulted in a higher incidence of graft rejection is because of the depletion of regulatory B cells.

Although alloantibodies have been known for their central role in provoking transplant rejection through complement-dependent and complement-independent pathways [63], the results in this thesis suggest that there is not an absolute correlation between graft rejection and IgG alloantibody production.

I have also established that IL-10 is not the major mechanism involved in T2-MZP B cell regulatory functions, in contrast to what has previously been reported in autoimmunity [146,149]. It is possible that in transplantation T2-MZP B cells exert their regulatory function through the involvement of other cytokines or ligands, however, further investigation is required to clarify this point.

Finally, I have shown that Gal-1 is essential for T2-MZP B cell regulatory function when isolated from mice housed in the CV facility. The mechanism behind the regulatory role of Gal-1 during transplantation remains to be investigated. However, as mentioned earlier in the discussion there might be more than one possible mechanism: for instance the effect of Gal-1 on B cell development as there was a significant difference in the percentages of T2-MZP B cells between WT and Gal-$1^{-/-}$ mice. Moreover, Gal-1 might be required in T2-MZP B cells signaling pathways, as there were significant differences in NF-κB translocation, P38 and ERK phosphorylation between Gal-$1^{-/-}$ and WT B cells. In addition, Gal-1 production by T2-MZP B cells might be essential in shifting T cell responses from Th1 and Th17 into Th2. It might also be due to the involvement of Gal-1 in the immunological synapse between T and B cells,

as Gal-1 has been reported to bridge linking cells to each other and cells to their extracellular matrixes [174].

Linked to one of the possible mechanisms that explain the loss of regulatory ability when Gal-1 is absent in B cells, I have for the first time identified the importance of Gal-1 expression in TLR-4 and 9 signaling in B cells. Here, I found that Gal-1 up regulates NF-κB and P38 pathways, and down regulates ERK pathway in B cells in response to TLR-9 ligand. This regulation provided by Gal-1 is essential in controlling IL-10 production in B cells. However, in the case of TLR-4 ligation, the high IL-10 production was only correlated with the decrease in NF-κB translocation, but no obvious involvement with ERK or P38. These findings suggest that Gal-1 plays different regulatory role in controlling IL-10 production by activated B cells depending on the TLR that has been ligated. However, how Gal-1 provides this role and where Gal-1 is involved in these pathways still needs to be investigated.

As mentioned earlier in the Introduction Chapter (section1.2.1), T2-MZP B cells are immature cells that might, upon adoptive transfer or co-culturing *in vitro* with T cells, mature into other subsets. However, its difficult to prove whether T2-MZP Breg cells represent a stage of B cell maturation or a subpopulation of B cells on their own. Therefore, thinking of a therapeutic use of these cells to induce tolerance is at this stage difficult. The expansion of T2-MZP B cells *in vitro* for adoptive cell therapy would be a complicated process unless the right stimuli and conditions are established to yield and maintain regulatory B cells with a T2-MZP B cells phenotype and properties. Alternatively strategies should be applied to increase the number of these cells directly *in vivo*. Whether

cellular therapy with Tregs, which is already in use in the clinic, can achieve the expansion of Bregs *in vivo* will be an important question to investigate when Treg cellular therapy is in routine use.

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